Sources, transformations, and fates of riverine organic matter

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Sources, transformations, and fates of riverine organic matter

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presented by
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Summary

In my Ph.D. research I have investigated the sources, transformations, and fates of riverine organic matter along the Tagliamento River, northeast Italy. It is a 7th order braided river grading from the Carnian Alps to the northern Adriatic Sea and regarded as the last large natural river Europe’s. This thesis work ties detailed chemical and molecular characterization of organic materials to its photochemical and biological reactivity, to better understand the importance and magnitude of riverine processes for the global carbon cycle. Riverine transformations are particularly important because they link terrestrial and marine environments.

Elemental and stable isotopic compositional indicators reveal that woody debris and dissolved organic matter (DOM) are the two major important pools of carbon and energy resources in the Tagliamento River. However, while woody debris comprises a slowly decomposing standing stock of organic matter, DOM summarizes a diagenetically aged compartment of high-molecular weight (HMW) and hydrophobic substances (20 – 40%), and a young and fast cycling compartment of low-molecular weight (LMW) compounds with dominant hydrophilic character (60 - 70%). Additional chemical and molecular characterization of HMW and hydrophobic DOM isolates utilizing NMR experiments and GC/MS analyses helped to delineate different origins of these materials and to understand their biological and photochemical reactivity. HMW and hydrophobic DOM predominantly derive from vascular plant material, soil biomass and to a lesser extent from microbial production in the river. Conversely, LMW and hydrophilic DOM seem to derive from decomposition of suspended organic particles and autochthonous microbial production. Over the year, downstream processing of LMW and hydrophilic DOM is fast and efficient compared to HMW and hydrophobic DOM, thereby, creating a uniform spatial-temporal chemical composition.

Photochemical and iron-catalyzed transformations are shown to strongly contribute to downstream DOM processing. Sunlight severely impacts the short- and long-term bacterial utilization of HMW and LMW as well as hydrophobic and hydrophilic DOM fractions. Apparently, light-induced production of reactive oxygen species in the presence of abundant iron causes a short-term inhibition of bacterial DOM
utilization. In addition, photochemical alteration of LMW and hydrophilic DOM structures causes a long-term (~days) inhibition of their bacterial utilization.

In the Tagliamento River, microbes living planktonic or surface-attached dominantly utilize LMW and hydrophilic DOM. Short turnover times (6 – 11 days) for these compounds compared to fast surface flow from the headwaters to the river estuary (2 - 3 days) imply that almost half of this material can be consumed during downstream transport. HMW and hydrophobic DOM exhibit much higher turnover times (110 – 204 days), and thus, seem to be dominantly transported by surface flow to the Sea. Microbial decomposition of DOM leads to a release of very small and possibly biorecalcitrant organic moieties and inorganic nutrients. However, although the turnover of bioavailable materials is fast and nutrients are efficiently recycled in this cold alpine environment, the concentrations of DOC and dissolved inorganic phosphorus are very low, and bacterial activity is heavily limited by low temperature and the availability of nutritional resources. We show that this multiple limitation causes a high similarity between planktonic and attached bacterial communities. Small coccoid-shaped cells opportunistically explore this complex and heterogeneous riverine environment for scarce resources. Surface-attachment proves to be advantageous for bacterial growth and stimulates the formation of large exopolymeric structures. However, it appears that cells only reach low levels of complexity in biofilm formation and live either planktonic or surface-attached to cope with these highly limiting conditions.

These studies demonstrate that depending on its origin, age, and therefore diagenetic state, organic matter undergoes multiple transformations in the Tagliamento River. They also show that a semi-natural river has a high capacity to retain organic materials and to rework them to mainly small and recalcitrant organic moieties. Compared to other streams of that order, little amounts of compounds with high degree of alteration are transported to the marine environment, and due to their small size may escape precipitation. Therefore, we suspect that these compounds may contribute to the pool of old dissolved organic carbon in the wide realms of the deep ocean.
Zusammenfassung


Indikatoren, wie die elementare Zusammensetzung sowie die Signatur stabiler Isotopen von organischen Substanzen, zeigen, dass Totholz und gelöstes organisches Material die beiden wichtigsten Quellen von Kohlenstoff und damit die Hauptenergiequelle im Tagliamento Fluss ausmachen. Während jedoch akkumuliertes Totholz langsam dekompostierende Biomasse umfasst, setzt sich DOM einerseits aus diagenetisch veränderten hochmolekularen und hydrophoben Substanzen (20 – 40%) und andererseits aus frisch produzierten, sich schnell umsetzenden niedermolekularen Substanzen mit vorwiegend hydrophilem Charakter (60 - 70%) zusammen. Hochmolekulare und hydrophobe Substanzen wurden mithilfe von Ultrafiltration und Extraktion an Festphase isoliert und aufkonzentriert und deren chemische und molekulare Zusammensetzung unter Anwendung von NMR und GC/MS aufgeschlüsselt. Diese Untersuchungen halfen, die verschiedenen biologischen Quellen dieser beiden Fraktionen zu identifizieren und ihre photochemische und biologische Reaktivitaet zu verstehen. Hochmolekulare und hydrophobe Substanzen lassen sich auf Pflanzenmaterial, Bodenbiomasse und auf mikobielle Biomasse, welche im Fluss produziert wird, zuehren. Im Gegensatz dazu stammen niedermolekulare und hydrophile Substanzen aus der Dekomposition von suspendierten Partikeln und autochthonem Material. Unabhängig von der Jahreszeit und vom Flussabschnitt finden wir, dass
niedermolekulare und hydrophile Substanzen schnell transformiert werden, was zu einer einheitlichen chemischen Signatur von DOM im Flusswasser führt.


Diese Studien zeigen, dass abhängig von Material, Alter, und damit vom diagenetischen Zustand, organische Substanzen im Tagliamento unterschiedlichsten Transformationen unterliegen koennen. Dieses natürliche System kann organisches Material effizient retentieren und einen Umbau zu kleineren, refraktären Substanzen
bewirken. Damit werden im Vergleich zu anderen Flüssen dieser Größe eher geringe Mengen an organischem Material zum Meer transportiert. Wir nehmen an, dass diese niedermolekularen und stark refraktären Substanzen beim Eintrag in Salzwasser kaum ausgefällt werden und damit zum marinen Pool an alten, niedermolekularen, refraktären Substanzen beitragen.
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This work is dedicated to my Mum and Dad, who grew me up, who taught me to love life and be respectful, and who love me enough to let me go my own way.
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g. KAISER, E., AND B. SULZBERGER. Decomposition of riverine organic matter in the presence of ambient and increased dissolved iron concentrations.
General Introduction

The importance of rivers for the global carbon cycle

Rivers are important freshwater systems that link the carbon cycle of the continents to that of the oceans (Berner 1989). They vitally integrate terrestrial and marine reservoirs of carbon energy resources and their transformation processes. River systems for example discharge sufficient organic material (approximately $0.4 \times 10^{15}$ g C yr$^{-1}$) to account for the global turnover of dissolved organic carbon (DOC) in the vast oceanic realm, and for the temporary removal of organic carbon through marine sedimentation (Williams and Drueffel 1987; Berner 1989; Hedges and Keil 1995). However, global estimations for riverine fluxes partly arose from modified and regulated river systems. Human impacts are many-fold (Petts 1989), and have generally affected the total water discharge and threatened floodplain habitat diversity (Muhar 2000). For this reason, we have great need to study the processing of organic compounds during transport in highly variable and dynamic natural systems. This knowledge will contribute to a better understanding of the global carbon cycle over geological time-scales.

Organic carbon is essential to all life processes and vital for the flow of energy through aquatic and terrestrial environments (Wetzel 1992). Dissolved organic matter (DOM) represents a major reactive reservoir of reduced organic carbon on Earth (Schlesinger and Melack 1981; Meybeck 1981; Hedges et al. 1997). Therefore, the questions of not only the transport but also the transformations of riverine DOM are key to understand the biogeochemical cycling of carbon (Berner 1989). Rivers alter and solubilize organic matter through biological, chemical, and physical reworking (Ittekot 1988; Hedges et al. 1994; Raymond and Bauer 2001). Because origin and molecular features of organic matter are crucial for its overall reactivity within system processes (Wetzel 1992; Hedges et al. 1992, 1997; Findlay and Sinsabaugh 1999), elucidating organic matter molecular composition and structure is essential to understand its transformations within aquatic systems.

In my Ph.D. Thesis, the first three chapters present studies on the origin and the specific chemical features of organic matter in the Tagliamento River. Chapters four and five elaborate on different transformation pathways and possible fates of riverine organic
matter. Each chapter is in the format of a scientific publication (see list of publications). Some of them also contain information that will be integrated into two future publications (list of publications).

**Thesis goals**

The motivation for this thesis arose from the need to better understand the processing of different organic matter resources in a natural and highly heterogeneous riverine environment. The Tagliamento River in northeast Italy is regarded as the last large natural and morphologically intact river in Europe (Mueller 1995; Ward et al. 1999). Its river corridor connects the Southern Alps with the northern Adriatic Sea and grades from headwater tributaries to braided floodplains, and a lowland meandering reach near the river mouth (Ward et al. 1999). The channel structure is highly complex and the channels shift regularly and dramatically in response to peaks in the hydrograph (Arscott et al. 2002).

Chapter one is dedicated to elucidating different origins of organic matter, in particular of fine suspended organic particles and DOM. Compositional indicators, such as elemental carbon:nitrogen ratios and stable isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$), were used for source assignments and the description of the diagenetic state of DOM. Several preceding studies have applied these methods (Meybeck 1981; Hedges et al. 1986; 1994; Finlay 2001), however, this is the first time that different DOM isolates have been compared to a variety of particulate organic materials to delineate source compounds. Further, the abundance and distribution of inorganic nutrients in mainstem waters helped to estimate organic matter transformations and fates.

The chapters two and three report an extensive spatial-temporal characterization of the chemical and molecular composition of different DOM isolates (HMW and hydrophobic DOM). The application of solid-state $^{13}$C NMR and multidimensional solution-state NMR experiments allowed studying complex DOM structures. Several studies from the literature have used these approaches (Hedges et al. 1992; Lampert et al. 1992; McKnight et al. 1997), but this is the first time that different riverine DOM isolates were utilized for multidimensional solution-state NMR experiments. Further
characterization with TMAH-GC/MS added detailed information on DOM molecular composition and helped to identify potential source compounds. Size exclusion chromatography allowed characterizing LMW and hydrophilic DOM, that escape concentration and isolation due to their low carbon and high salt content. To better understand DOM transformations along the river continuum, we also measured the bioavailability of HMW, LMW, hydrophobic, and hydrophilic DOM. Bacterial DOM consumption is recognized as an important indicator for DOM reactivity, transformations, and fates, since microorganisms are the major consumers of DOM in aquatic systems (Cole et al. 1988), and key for the biogeochemical cycling of DOM in rivers (Findlay et al. 1991; 1992; Leff 1991).

Chapter four reports that photochemical transformation of DOM in the presence of abundant iron strongly impacts bacterial DOM processing, because it causes the production of reactive oxygen species and alters the chemical signature of DOM components. In the last two decades the importance of photochemical transformations for the cycling of carbon in aquatic systems has been recognized and intensively studied (Kieber et al. 1989; Mopper et al. 1991; Smith et al. 1989; Benner and Biddanda 1998). However, studies on rivers are rare, especially on semi-natural environments like the Tagliamento River. The specific goals of the study presented in this chapter were (i) to determine the short- and long-term impact of solar radiation on freshwater DOM and its bacterial utilization, (ii) to assess the photoreactivity of these DOM fractions towards Fe(II) and H$_2$O$_2$ formation, and (iii) to investigate the chemical composition of DOM fractions before and after phototransformations and relate it to their bioavailability.

Chapter five elaborates on the different life strategies of riverine bacteria that cope with low temperatures as well as low DOC and dissolved inorganic phosphorus concentrations. We compare the activity, community structures and growth forms of planktonic and surface-attached cells. Because habitat heterogeneity was shown to severely determine microenvironments in riverine ecosystems (Geesey et al. 1978; Arscott et al. 2000), we were interested which different strategies for survival may be provoked by the heterogeneous Tagliamento River.
Literature Cited


CHAPTER 1

Transformations and discharge of organic carbon and nitrogen in the Tagliamento River, Italy

KAISER, E, D. B. ARSCOTT, K. TOCKNER, AND B. SULZBERGER

Living wood and leaf biomass, woody debris, fine soil particulate organic matter (FSPOM), fine suspended organic particles (FPOM), and solid phase extracted and ultrafiltered dissolved organic matter (DOM) were seasonally collected in a longitudinal direction from the last large natural river Europe’s, the Tagliamento (Italy). Elemental carbon (C) and nitrogen (N) concentrations, and stable C and N isotopic compositions (δ13C and δ15N) are reported for the individual POM and DOM samples. Downstream abundances and distributions of C and N were related to inorganic nutrient concentrations and discharge to infer transformations and transport of organic compounds. The C:N ratios and δ13C and δ15N values were used to identify potential sources for the different FSPOM, FPOM, and DOM samples. We found that FSPOM dominantly contains strongly diagenetically altered plant material and prokaryotic biomass. Suspended FPOM and solid phase extracted and ultrafiltered DOM is mainly comprised of river-borne bacterial and microalgal biomass and soil-derived organic matter. However, compared to DOM isolates, FPOM shows highest N content rendering it highly biologically reactive. Its biotic degradation and remineralization releases bioavailable compounds, possibly DOM differing in structure and molecular weight from the isolated DOM fractions, and nutrients (e.g. nitrate). We propose that turnover of FPOM is fast (<1 day) compared to woody material, FSPOM, and DOM isolates. Therefore, FPOM decomposition is major important for the formation of bioreactive DOM sources, and overall for the energy and nutrient flow in Tagliamento surface waters.
Introduction

C and N are essential to all life processes and vital for the flow of energy through aquatic and terrestrial environments (Wetzel 1992). POM and DOM represent major reactive reservoirs of organic C and N on Earth (Meybeck 1981; Hedges et al. 1997). Exploring their transport and transformations are key to understanding the global biogeochemical cycling of these important elements. Rivers are important aquatic systems that receive, produce, and discharge a significant portion of organic material. Their organic load may even account for the global turnover of dissolved organic carbon (DOC) in the vast oceanic regime (Williams and Drueffel, 1987).

Rivers are diverse freshwater ecosystems that integrate a wide range of different habitats, reaching from alpine regions to coastal and oceanic realms (Meybeck 1981; Hedges et al. 1997; Opsahl and Benner 1997). Over the last years we learned that these systems perform multiple functions, which heavily depend on climate, catchment area, habitat structure and diversity, trophy, and many others (Ward 1998; Arscott et al. 2000; Tockner et al. 2002). Therefore, rivers not only transport inorganic and organic matter, but transform it through chemical, physical, and biological reworking (Ittekot, 1988; Hedges et al., 1994; 1997; Raymond and Bauer, 2001). Particularly, the origin of material is of interest, because it determines its chemical reactivity and bioavailability to the aquatic microbial community, and induces specific transformation pathways (Wetzel 1992; Hedges et al. 1992; 1997).

Over the past three decades, applications of elemental ratios and stable isotopes for source identifications and process studies in terrestrial and aquatic environments proliferated. But, original distinct C:N ratios for different biomacromolecules that contribute to terrestrial or freshwater organic matter can be blurred by selective loss of either the one or the other element during degradative processes (Hedges et al. 1986b; 97). Thus, it is essential to combine this bulk compositional indicator with other chemical properties, such as stable C and N isotopic compositions. The $\delta^{13}$C of POC and DOC have been used successfully to identify organic C sources and enhance the understanding of organic matter cycling (Fogel and Cifuentes 1993; Coffin 1989; Peterson et al. 1994; Canuel et al. 1995; Hall 1995; Trumbore et al. 1995; Benner et al. 1997; Goni et al. 1997; Raymond and Bauer 2001). The $\delta^{15}$N have been beneficial to study the cycling of N, and
in particular processes, such as N$_2$-fixation, nitrification, and denitrification (Wada and Hattori 1976; 1978; Altabet 1988; Bronk et al. 1994; Benner et al. 1997). $\delta^{15}$N may also function as trophy indicators and help to trace the flow of N within food webs (Macko et al. 1983; Fry 1991; MacLeod et al. 1998). Many isotope composition studies had been conducted in freshwater or marine systems, but detailed information on how C and N cycle spatially and temporally while in riverine transport is still scarce (Finlay et al. 1999; 2001). There is further need to better understand the exchange of energy and nutrients between ecosystems, especially at the land-river interface.

The organic material borne by larger lotic systems is largely terrestrial POM and DOM (Meybeck 1981; Hedges et al. 1986b; Webster and Meyer 1997; Findlay and Sinsabaugh 1999; Raymond and Bauer 2001) and highly degraded (Ittekot 1988; Hedges et al. 1994). Owing to isotopic differences between C$_3$- and C$_4$-plants, different vascular plant sources (Fogel and Cifuentes 1993) as well as their turnover can be readily identified. Isotopic values of organic particulates were found to closely match that of soil organic matter (Meybeck 1981; Hedges et al. 1986b). However, depending on riparian vegetation, stream size, and discharge, autochthonous microbial production may also contribute significant portions to riverine organic matter (Minshall 1978; Vannote 1980; Finlay et al. 2001). The influx and presence of these different sources vary along longitudinal transport and also with seasons, mainly due to large spatial-temporal variations in physical and chemical characteristics (Hobbie 2000). Therefore, the isotopic signatures of the investigated materials may overlap to a significant degree, and the interpretation of $\delta^{13}$C and $\delta^{15}$N measurements for source identification in aquatic systems can be difficult.

In this study, the organic C and N concentrations and stable isotopic compositions of organic materials were measured to provide information about the origin and chemical and biological reactivity of riverine FSPOM, FPOM, and DOM. The samples were taken along the Tagliamento River, located in northeast Italy (Fig. 1.1). The measurements represent the first measurements of the stable N isotopic composition of riverine solid phase extracted and ultrafiltered DOM. Downstream inorganic nutrient concentrations in the Tagliamento mainstem and in the four major tributaries helped to elucidate sources of FSPOM, FPOM, and DOM isolates and permeates as well as their different
decomposition and remineralization pathways. The overall goal of this study was to investigate the origin, spatial-temporal transformations, and fates of organic substances that serve as a major important energy resource for the whole river ecosystem.

Materials and methods

Study area and hydrology

Since 1997 the TAGLIAMENTO project has been extensively studying the whole river corridor of the Tagliamento River, extending over 170 km (Ward et al. 1999; Gurnell et al., 2000; Arscott et al. 2000; Karrenberg et al. 2002; Nat van der et al. 2002; Kaiser et al. 2003a; 2003b). The river has been described as the last large semi-natural river in Europe (Mueller, 1995) flowing unrestrained from the alpine headwater reaches, situated in the limestone formations (carbonate, dolomite) of the Carnian Alps, through the Friulian Plain to the northern Adriatic Sea (Fig. 1.1). Mt. Colians is the highest peak in the catchment (2781 m above sea level (a.s.l.)). The mainstem of the river arises from a spring located at 1195 m (a.s.l.). Gypsum deposits are located up-gradient from the spring area. Reach morphology and channel structure changes along the river continuum, grading from headwater tributaries to braided floodplains, and a lowland meandering reach near the river mouth (Ward et al. 1999; Fig. 1.1). The channel structure is highly complex and the channels shift (via avulsion and lateral cut-and-fill erosion/deposition processes) regularly and dramatically in response to peaks in the hydrograph (Arscott et al. in press). The hydrograph is a flashy pluvio-nival regime driven by intense rainfall events in Autumn, and rain on snowpack in Spring. Flooding is not limited to spring and autumn, however, and rain events producing peak flow conditions have occurred in other seasons, as evidenced from the long-term hydrograph (Edwards et al. 1999; Ward et al., 1999; Campolo et al. 1999). Average discharge at river-km 59 (7th order) is 90 m$^3$ s$^{-1}$ with 2, 5, and 10 year return period floods estimated to be 1100, 1500, and 2150 m$^3$ s$^{-1}$ (Gurnell et al., 2000). Major tributary streams to the Tagliamento River are the Arzino, But, Degano, and Fella (Fig. 1.1).

At two locations along the Tagliamento mainstem the streambed typically lacks surface water during summer low-flow conditions. At the upstream location from
approximately river-km 38 - 43 the valley floor widens and alluvial deposits deepen, thus forming a downwelling zone, where surface water is naturally lost to subsurface or interstitial pore space. After river-km 43 groundwater upwelling and tributaries, such as the But and Degano, contribute water to the mainstem, thereby replenishing surface water. Additionally, water abstraction at river-km 33 exacerbates dry conditions downstream. At this point, water is pumped upslope and then released downslope to a reservoir for hydropower generation. The abstracted river water returns to the Tagliamento mainstem at river-km ~68 via the outlet of a natural lake (Lago di Carozzo). The second point along the continuum that lacks surface flow summer low-flow occurs naturally and is located from approximately river-km 104 - 110 (see Ward et al. 1999).

Figure 1.1
Catchment site of the Tagliamento River with important sampling stations marked by large grey patches: Main channel in headwater floodplain (R2MC), in major floodplain (R4MC), and in transition floodplain (R5MC), and isolated pool in major floodplain (R4P). Find the most important tributary streams, the Arzino, But, Degano, and Fella in the catchment area. The little inset shows that the river is located northeast Italy, Europe.
Sample collection

In order to determine longitudinal pattern in nutrient concentrations, water samples were taken at 3 – 5 km intervals along the entire length of the Tagliamento mainstem during summer low-flow (August 25 and 26, 2001). To estimate the extent of point and non-point source nutrient addition to mainstem surface waters, samples were also taken at 3 – 5-km intervals from the main channels of the tributaries Arzino, But, and Fella (Fig.1.1). The tributaries and the Tagliamento mainstem were near base-flow conditions during sampling. The pH, and temperature were measured in the field under in-situ conditions.

Particulate matter was collected by filtering 1 – 4 L of raw river water on GF/F filters. Te filters were stored frozen for particulate organic C (POC) and particulate N (PN) and particulate P (PP) analyses. Water for inorganic nutrient analyses and dissolved organic N (DON) were filtered using a disposable, sterile 0.45 μm cellulosenitrate prefilter (Sartorius) with a sterile 60 mL syringe (Merck), and the filtrates were stored in muffled 500 mL Schott-bottles (Duran) at 4°C in the dark. Subsamples for TOC and DOC analyses were taken directly after filtration in acid-rinsed, 20 ml PP tubes (Greiner), acidified to pH~1, sealed, and stored at 4°C.

Bimonthly particulate organic matter (POM) samples, such as woody debris, benthic algae, fine particulate material contained in terrestrial soil sediment (FSPOM), and sestonic particles >0.7 μm (FPOM), were collected along the main channel (MC) in the island-braided headwater floodplain (R2), the island-braided lower and major floodplain (R4), and in the braided-to-meandering transition floodplain (R5). Additionally, material was sampled from an isolated pool (P) located in R4 (Fig. 1.1, Table 1.1). Samples were collected on seven dates in: March, May, July, August, October, and November 1999 and April 2000. Living wood and leaf material (Salix eleagnos, Populus nigra, and Alnus incana) was additionally collected from R4 in April 2000. This material were packed in clean, acid-rinsed plastic-bags, sealed, and stored frozen for further analysis. POM was collected by filtering raw river water (1 - 4 liters depending on turbidity) through muffled GF/F filters (250 °C, Whatman). The filters were wrapped in muffled aluminium-foil, and stored frozen.

Water for C18-solid phase extraction and ultrafiltration were bimonthly sampled from the Tagliamento main channels in R2, R4, and R5, and from the pool in R4 during
the sampling months. Water levels during sampling varied from low- to high-flow conditions (Table 3). Water samples (~200 L) for DOM fractionation were collected with clean 50 L high-density polyethylene (HDPE) carboys and were transported back to the laboratory near R4 for further processing. Immediately following collection, water samples were passed through muffled GF/F- and 0.2 µm Durapore filters (142 mm diameter, Millipore) and stored in clean 50 L carboys. The carboys, the teflon-lined filtration unit, and the silicone and Tygon tubes were thoroughly cleaned with 1 M hydrochloric acid and MQ-UV water (Millipore) and were tested to behave non-contaminating for bulk DOM characterization.

**DOM fractionation and isolation**

The fractionation of all water samples was performed at a field station in Italy close to the Tagliamento in R4. DOM was either chemically fractionated or size fractionated. Only during the April 2000 sampling, DOM was collected by employing both methods in parallel for a more complete recovery of DOM components. Chemically fractionation of DOM into hydrophobic and hydrophilic compounds was achieved using a Mega Bond Elute C18 column (C18 loaded silica, 60CC, Varian) and by following the procedure of Louchouarn et al. (2001). From the bulk DOC (DOC <0.2 µm) 6 - 29% were recovered as a hydrophobic fraction after sorption, elution, and lyophilization (Table 3). Fractionating of bulk DOM into high- and low-molecular weight (HMW and LMW) DOM was achieved using a Filtron tangential flow ultrafiltration system with a polyethersulfone membrane (1 kDa nominal weight cutoff) and by following the protocol of Benner et al. (1997) and Kaiser et al. (2003b). Water temperature ranged from 20-22ºC during ultrafiltration. Recovery of ultrafiltered DOC (cDOC) varied between 4 - 9% of bulk DOC after concentration and freeze-drying (Table 3). For every sample, a C mass balance was established to determine whether C was lost or gained during fractionation. Samples for DOC analysis were collected directly after fractionation in acid-rinsed, muffled 40 ml EPA glass vials, acidified to pH~1 (2 M HCL), sealed with Teflon-lined caps, and stored frozen until analysis. The percentage of DOC recovered after fractionation from bulk DOC was calculated as follows: % of initial DOC (<0.2µm) = 100 (DOC_{retentate} + DOC_{permeate}) / (DOC_{<0.2µm})^{-1}, where DOC stands for DOC concentration and was corrected
by the according concentration factor (Table 1.3). Mass balance calculations revealed that from initial DOC 99 – 144% were recovered after ultrafiltration and 100 – 139% after C18 solid phase extraction.

All samples for laboratory experiments were stored at 4°C in the dark and immediately brought back to EAWAG Zurich, Switzerland for further analysis. DOM adsorbed onto the C18 phase was eluted by gentle vacuum-filtration using high-purity methanol (Merck). The methanol phase was removed from DOM by roto-evaporation and freeze-drying. The extract was then again redissolved in MQ-UV water (Millipore). For removal of trace metals for $^{13}$C NMR experiments, two solid phase extracted and one ultrafiltered DOM sample were cation-exchanged (Bio-Rad, AG 50W and AG MP-50, Everett et al. 1999). For further chemical characterization all DOM concentrates were freeze-dried to a powdery solid phase. The dried material were scraped from the Teflon-beaker and stored sealed in muffled glass tubes (Supelco) at 4°C in the dark for elemental, stable isotope, and $^{13}$C NMR analyses.

**Measurements of organic C and N and inorganic nutrients**

For elemental and stable C and N isotope analyses, woody debris, fresh wood and leaf material, and FSPOM were freeze-dried and homogenized to a powdery state. Then, homogenized POM and FPOM on GF/F filter was acidified by vapor-phase to remove residual carbonates. The DOM isolates were already recovered in a solid powdery phase (see above). The C and N content of the various POM and DOM samples were measured by using a Vario-EL CHNS analyzer (Hedges and Stern 1984) (Tables 1.1 – 1.3).

Total organic C (TOC) and DOC concentrations were determined by high temperature catalytic oxidation with a Shimadzu 5050A analyzer (Benner et al., 1993; 1997). POC was calculated as the difference between TOC and DOC concentrations. For samples from August 2001, concentrations of ammonium ($\text{NH}_4^+$), nitrite ($\text{NO}_2^-$), nitrite plus nitrate ($\text{NO}_2^- + \text{NO}_3^-$), total dissolved nitrogen (TDN), dissolved organic nitrogen (DON), soluble reactive phosphorous (SRP), total dissolved phosphorous (TDP), particulate nitrogen and phosphorous (PN, PP) were analyzed as reported in Arscott et al. (2000). Particles collected on GF/F filters were not acid-fumed and may have contained inorganic N and P, in contrast to seasonally collected particles, which were treated with
acid. Therefore, we used the notation PN and PP for August 2001 samples. Nitrate (NO$_3^-$) was calculated as ([NO$_2^-$] + [NO$_3^-$]) - [NO$_2^-$], DON was calculated as TDN - ([NO$_2^-$] + [NO$_3^-$]) - [NH$_4^+$]. Temperature (± 0.1°C) and pH (± 0.01) were measured in the field using a Universal Pocket Meter Multiline P4 (WTW GmbH).

**Stable C and N isotope analyses**

Stable C and N isotope compositions of dried and homogenized POM and isolated DOM were measured on an Isoprime isotope ratio mass spectrometer (Micromass). Samples were combusted at 850°C. Isotopic compositions are reported as

$$\delta^N = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1,000,$$

where $^N$ is the heavy isotope of an element (13C or 15N) and $R$ is the ratio of 13C:12C or 15N:14N in the sample and the standards (Craig, 1957). The 13C and 15N isotope ratios of the samples were determined by comparison with the standards carbonate (NBS19 carbonate), sucrose (IAEA-CH-6), calcite (IAEA-CO-8), ammonium sulfate (IAEA-N-1, IAEA-N-2), potassium nitrate (IAEA-NO-3), and lithium carbonate (LSVEC), with all values reported relative to the PeeDee Belemnite and atmospheric N$_2$ standards. The reproducibility of analysis was typically ± 0.1‰ for $\delta^{13}$C and ± 0.3‰ for $\delta^{15}$N.

**Solid-state 13C NMR spectroscopy**

13C NMR spectra of dried and homogenized POM and DOM were obtained as described by Zang et al. (2000) and Dria et al. (2002), using the ramp cross polarization magic angle spinning (ramp CPMAS) pulse program and two pulse phase modulated (TPPM) decoupling on a Bruker DSX 300 NMR spectrometer, operating at a frequency of 300 MHz for 1H or 75.48 MHz for 13C. Approximately 20 - 30 mg of sample was spun at a frequency of 13 kHz using a contact time of 2 ms and a 1 s recycle delay time. For each sample 80,000 - 100,000 acquisitions (scans) were collected. For each sample’s free induction decay, 1,024 complex data points were collected and zero-filled to a total of 4,096 data points. The samples were Fourier-transformed, 100 Hz line-broadening was applied and phased appropriately. The carboxyl carbon of glycine (176.03 ppm) provided
a secondary reference for all solid-state spectra. The spectra were split in aliphatic and aromatic regions and integrated over 0 - 60, 60 - 90, 90 - 110, 110 - 160, 160 - 180, and 180-230 ppm regions for quantitative comparisons. The aliphatic region includes the integrals between 0 - 110 ppm, and the area between 110-160 ppm was assigned to aromatic carbons (Malcolm, 1990). The aromaticity and aliphaticity of each sample was calculated as a percent of the total integrated area.

**Results**

**Downstream speciation and concentrations of dissolved nutrients**

From bimonthly sampling, mainstem river water was characterized by 10.9 ± 2.5°C, by pH 8.13 ± 0.14, and an alkalinity of 2.8 ± 0.5 mM (± standard error, SE). During August 2001, similar values were found (Fig. 1.2 A). Limestone formations (carbonate, dolomite) and gypsum deposits located up-gradient from the spring area contributed to stable and high pH and alkalinity (data not shown).

During the field study in August 2001, dissolved inorganic N was abundant in the Tagliamento mainstem, increasing from the source region to lower reaches (river-km 168), with nitrate as the dominant nutrient species present. Nitrate increased from ~18 - 89 µM N and ammonium from ~0.2 - 3 µM N. The increase in nitrate in the lower section of the river (river-km 120) was due to elevated nitrate in a groundwater fed tributary (Varmo River) and increased density of agriculture (Fig. 1.2 D). TDP was generally very low in the Tagliamento mainstem (Fig. 1.2 E), with an average value of 0.12 ± 0.02 µM P for TDP, and 0.03 ± 0.01 µM P for SRP. The larger standard errors were caused by two point-source additions of TDP and SRP at river-km 8.7 (local village effluent) and 104 (agricultural inputs and very low surface discharge for dilution). However, similar to PN and PP, additional TDP was quickly removed within a downstream distance of 3 km, attributable to receding surface flow and high retention of nutrients by the hyporheic zone. Other than these minor inputs, concentrations were uniformly low in the main channel.
Figure 1.2
Profiles show data collected from the mainstem of the Tagliamento River during August 2001: (A) temperature and pH, (B) PP and PN, (C) TOC and DOC, (D) TDN, DON, nitrate, and ammonium, and (E) TDP and SRP.
The tributaries (Fig. 1.3) generally increased in temperature downstream from their respective sources. Variations in temperature and pH in these headwater tributaries could be attributed to small stream sizes, larger variations in the surface flow regime, exposure to sunlight/shading, and biological activity. Fig. 1.3 summarizes the chemical speciation and the downstream distribution of inorganic nutrients in surface waters from three important tributaries to the Tagliamento River. TDN concentrations were lower than in the Tagliamento, but increased with distance (primarily driven by nitrate concentrations). Average (± SE) nitrate and ammonium concentrations for the tributaries were 43.95 ± 2.69 µM N and of 1 ± 0.23 µM N, respectively. TDP and SRP concentrations (0.19 ± 0.02 µM P for TDP and 0.01 ± 0.01) µM P were approximately double of what we measured along the Tagliamento mainstem, and showed large variations. Variations in dissolved nutrients tended to co-vary with PN and PP concentrations, suggesting that TDP is primarily released due to particle decomposition and remineralization.

Figure 1.3 Profiles show data collected from the mainstem of three tributaries to the Tagliamento River during a field campaign in August 2001: Fella, But, and Arzino.
Abundance and distribution of POM

POC concentrations of woody debris ranged from 30 to 270 µmoles C mg$^{-1}$ dry mass with an average (± SE) value of 67.51 ± 10.18 µmoles C mg$^{-1}$ (Table 1). There were no observable downstream pattern in concentration, however, there were seasonal trends. Concentrations were highest in March, strongly decreased until May 1999, stayed low until November, and then increased in April 2000. PON concentrations followed a similar seasonal pattern. PON concentrations ranged from 0.09 to 3.34 µmoles N mg$^{-1}$ with an average (± SE) value of 0.62 ± 0.15 µmoles N mg$^{-1}$ (Table 1.1). From the data it appears that N is selectively removed relative to C from March to November 1999. Likewise, POC and PON concentrations from non-decomposed and living wood and leaf biomass contained 75.66 ± 3.66 µmoles C mg$^{-1}$ and 0.59 ± 0.05 µmoles N mg$^{-1}$ (Table 1.1).

In the Tagliamento, the density of benthic algae was generally low but highly variable in time and space (unpublished data). Algal POC concentrations ranged from 5.85 to 153.74 µmoles C mg$^{-1}$ (55.47 ± 14.43 µmoles C mg$^{-1}$) (Table 1) with lowest values were measured in May and July 1999, and highest values in March 1999 and April 2000. PON concentrations ranged from 0.09 to 10.45 µmoles N mg$^{-1}$ (3.27 ± 1.09 µmoles N mg$^{-1}$) with lowest values in July 1999, and highest values in March 1999 and April 2000 (Table 1). POC and PON measured from FSPOM, sampled at the land-water interface, ranged from 0.37 to 18.37 µmoles C mg$^{-1}$ (9.47 ± 4.51) and from 0.02 to 0.36 µmoles N mg$^{-1}$ (0.18 ± 0.07), respectively (Table 1.1).

In August 2001, organic particle load was small in the Tagliamento mainstem, and increased in a downstream manner (Fig. 2 B, C). Longitudinal variation in POC (14 ± 1.1 µM C) was less than in PN (0.76 ± 2.6 µM N) and PP (0.16 ± 0.52 µM P) and followed DOC dynamics. Nutrient concentrations revealed point-source additions at river-km 8.7, 55, and 104, which overlapped with peaks in TDP and SRP concentrations (Fig. 1.2 D, E). Increased concentrations of DOC, PN, and PP at river-km 55 resulted from pulp mill effluents. Shortly downstream DOC, PN, and PP concentrations were diluted to upstream levels, due to flow from the Fella tributary (carrying a greater discharge than in the mainstem). We detected another peak in PN and PP after river-km 140 that was associated with a peak in SRP and TDP. Particle export from the three tributaries to the Tagliamento was low with average (± SE) inputs of 14 ± 1.1 µM C for
POC, of 1.68 ± 0.19 µM N for PN, and of 0.16 ± 0.52 µM P for PP (Fig. 1.3). Dynamics reflected patterns in TOC, DOC, TDP, and SRP concentrations.

During bimonthly sampling in the Tagliamento River, the recovery of organic particles was calculated as the percentage of POC from TOC concentrations. POC concentrations of FPOM ranged from 0.1 to 19.9% of the TOC (5 ± 1%) along the Tagliamento mainstem (Table 1.2). In most cases, downstream samples had greatest particle loads. POC and PON concentrations of FPOM (from elemental analysis) were highly variable (Table 2). POC ranged from 0.21 to 12.57 µM C (3.47 ± 0.75 µM C). PON was abundant relative to POC. PON ranged from 0.05 to 6.84 µM N (1.76 ± 0.42 µM N). In contrast to wood material and benthic algae biomass, there were no seasonal, but longitudinal changes in POC and PON concentrations. Highest concentrations were observed in R2 and R4, and lowest concentrations in R4P (Table 1.2).

**Abundance and distribution of bulk DOM**

In August 2001, the abundance of TOC and DOC along the Tagliamento mainstem was uniformly low, with the exception of the point-wise pulp mill influx mentioned previously and with averages (± SE) of 101 ± 0.1 µM C and 91 ± 0.2 µM C, respectively (Fig. 1.2 C). DON increased downstream from ~5 to 33 µM N with downstream transport (15.1 ± 0.9 µM N) (Fig. 2 D). The three tributary streams averaged inputs of 82.4 ± 4.5 µM C for TOC, 75.9 ± 3.3 µM C for DOC, and 11.3 ± 0.5 µM N for DON to the Tagliamento mainstem (Fig. 1.3).

Bimonthly measurements of DOC concentrations in surface waters ranged from 32 to 95 µM C (64.48 ± 3.84 µM C) (Table 1.3). Concentrations were always highest in R4MC and lowest in R4P and R5MC. Over the year, DOC concentrations were highest in March 1999 and April 2000, and correspond to high-flow. In July 1999, discharge was closer to base-flow level and DOC values were low. Hence, during summer the river system became very homogeneous, exhibiting almost equal low amounts of DOC present in all reaches. During high-flow conditions in August (post-flood) and November 1999 we measured a nominal increase in DOC. This August flood event disrupted summer low-flow and low DOC concentrations and supplied a short-term spike of C to the system. During post-flood conditions DOC concentrations were highest in R2MC.
Abundance and distribution of DOM isolates

The isolation and concentration of riverine DOM to a solid matter allowed to analyze its elemental C and N content. The percentage of surface water DOC retained by C18-solid phase extraction (cDOC) ranged from 6 to 29% (15.7 ± 1.7%) and by ultrafiltration ranged from 6 to 9% (6.3 ± 0.4%) (Table 1.3). The amount of material isolated by both techniques was overall low compared to literature values (Table 3) (Amador et al. 1990; Benner et al. 1997). Incomplete elution from the C18-column and high concentration of ultrafiltration retentate volumes may explain this result (Benner et al., 1997). Further, the Filtron ultrafiltration system has been shown to retain DOM with lower efficiency (Buesseler et al. 1996) than the commonly used Amicon system (Benner et al. 1997). Despite adjustments of water flow and pressure conditions to obtain optimal recovery, however, recovered amounts were still less than by other ultrafiltration systems.

Concentrations of cDOC were variable throughout the year and ranging from 2.9 to 15.9 µM C (8.40 ± 0.98 µM C) for solid phase extracts and 2.4 to 7.3 µM C (4.61 ± 0.38 µM C) for ultrafiltered isolates (Table 1.4). Longitudinal trends were not evident, however, solid phase extracted cDOC exhibited highest concentrations in March 1999 and April 2000, and lowest values in July 1999. The pattern was less pronounced for ultrafiltered cDOC. Conversely, concentrations of cDON were highest in October and November 1999, ranging from 0.13 to 0.43 µM N (0.23 ± 0.02 µM N) for solid phase extracts and 0.06 to 0.25 µM N (0.16 ± 0.02 µM N) for ultrafiltered isolates (Table 1.4).

C:N ratios, stable isotopic compositions, and bulk chemical compositions of POM and DOM

C:N ratios of woody debris ranged from 49.2 to 357.3 (167.5 ± 15.8) (Table 1.1). Ratios were consistently highest in either R2MC or R5MC and lowest in R4P, indicating differences in composition and diagenetic state of the organic material present in the mainstem versus the isolated pool. Ratios were highest in March 1999 and April 2000, and slightly lower during the rest of the year, suggesting a selective loss of N during Summer, Fall, and Winter. In general, the ratios of living wood and leaf biomass (137.7 ± 12.9) were close to the average ratio of woody debris.
C:N ratios of benthic algae biomass ranged from 9.2 to 179.2 (46.2 ± 15.4) (Table 1.1). No longitudinal or seasonal pattern was observed, however, sample number was too low to infer any reasonable seasonal trend. C:N ratios of FSPOM were also variable and ranged from 16.3 to 90 (43.9 ± 16.3). Ratios appeared to follow the pattern found for woody debris, but again the number of samples was low and only inferences could be drawn from these few data points. C:N ratios of FPOM ranged from 0.2 to 18.5 (3.7 ± 0.9) (Table 1.2), which was lowest among all organic materials collected from the Tagliamento River. They generally decreased in a downstream manner and reached highest values in spring and early summer, whereas lowest values in late Summer and Fall.

C:N ratios of bulk DOM, sampled in August 2001, ranged from 1.7 to 36.5 (6.9 ± 0.1). In comparison to bulk DOM, the C:N ratios of surface water DOM isolates (cDOC and cDON) were higher (Table 4). Ratios of solid phase extracted DOM ranged from 20 to 69 (39.2 ± 4.6) and of ultrafiltered DOM from 15 to 58 (33.8 ± 3.7). Solid phase extracted DOM had elevated C:N relative to ultrafiltered DOM, indicating a difference in the bulk chemical composition of these two DOM isolates (Kaiser et al., 2003a).

Stable C and N isotope compositions of POM and DOM exhibited no longitudinal fractionation (Table 1.1, 1.2, 1.4; Fig. 1.4). Seasonal fractionation was also negligible, except for C isotopes from woody debris. All average δ values reported here are averages (± SE) of bimonthly measurements. We measured an average δ¹³C value of –30.1 ± 0.4‰ and δ¹⁵N value of –6.2 ± 0.4‰ for woody debris, and an average δ¹³C value of –34.4 ± 0.7‰ and δ¹⁵N value of –5.2 ± 1.3‰ for living wood and leaf material. The δ¹³C for woody debris declined by a maximum of 7‰ from March 1999 to April 2000. Benthic algae were also depleted in heavy isotopes and revealed an average δ¹³C value of –33.6 ± 1.7‰ and δ¹⁵N value of –3.1 ± 0.9‰. FSPOM and FPOM were isotopically heavier than all the other samples. FSPOM averaged a δ¹³C value of –27.4 ± 1.1‰ and a δ¹⁵N value of –1.7 ± 0.7‰. FPOM averaged a δ¹³C value of –27.4 ± 0.2‰ and a δ¹⁵N value of –0.9 ± 0.2‰. Solid phase extracted and ultrafiltered DOM exhibited a large difference in δ¹³C (~10‰). Solid phase extracted averaged a δ¹³C value of –28.1 ± 0.7‰ and a δ¹⁵N value of –3.9 ± 0.2‰, whereas ultrafiltered DOM averaged a δ¹³C value of –37.7 ± 1.1‰ and a δ¹⁵N value of –3.7 ± 0.5‰. We also tested the C18 resin for possible
contamination of the C isotopic signature, and found that it had no impact on the $\delta^{13}C$
values averaging $-38.5 \pm 0.1\%$ (Fig. 1.4).

The solid-state $^{13}C$ NMR spectra of woody materials, benthic algae, and DOM isolates exhibited four major functional groups of organic compounds: aliphatics, carbons adjacent to O or N (such as in carbohydrates, amino acids, esters, alcohols, etc.), aromatics, and carboxyls-/aliphatic amides. The chemical shift of more defined signals indicated specific functional groups or structures, such as: Alkyl groups (12-25 ppm), methylene (29-35 ppm), sugars, aliphatic methine, and methoxy-like $\alpha$–amino acids and alcohols (45-90 ppm), possible anomeric sugars (90-110 ppm), aromatics and alkenes (110-140 ppm), aromatic heterocycles (140-160 ppm), carboxylates, aliphatic amides (160-190 ppm), carbonyls and ketones (190-230 ppm).

The $^{13}C$ NMR spectra of POM and DOM samples showed broad resonances (Fig. 1.5), and only the DOM isolates (Fig. 1.5 D - F) had relatively low signal-to-noise ratios due to low sample quantities, sample complexity, and low organic C contents. Spectra D – F exhibited some sharp peaks that are uncharacteristic of DOM and may be attributed to contaminants. The sharp clipped peaks observed in the solid phase extracted DOM spectra D and E (~33 ppm) may be attributed to C18 contamination during the solid phase extraction process. The 0 ppm peak observed in the ultrafiltered DOM spectrum F is likely attributed to methyl siloxanes of undetermined origin.

Benthic algae contained the highest percentage of aliphatics (86%), followed by Salix eleagnos (fresh wood and leaf biomass) (73%), woody debris (72%), solid phase extracted DOM from April 2000 (68%) and March 1999 (61%), and ultrafiltered DOM from April 2000 (57%). In contrast, the portion of aromatics was highest in solid phase extracted DOM from March 1999 (28%) followed by ultrafiltered DOM from April 2000 (26%), woody debris (21%), Salix eleagnos (19%), solid phase extracted DOM from April 2000 (18%), and benthic algae (9%). The resulting aliphatic:aromatic ratios were 9.8 for benthic algae, 3.8 for woody debris, and 3.5 for Salix eleagnos. Solid phase extracted DOM from March 1999 and April 2000 exhibited reduced ratios of 2.2 and 3.8, and ultrafiltered DOM from April 2000 was characterized by a ratio of 2.2.
Figure 1.4
Average stable C and N isotopic compositions of POM and DOM isolates. Isotopic C and N ratios for C18-solid phase material prove that chemical fractionation did not contaminate nor shift the isotopic ratios of DOM isolates. Data represent means ± standard errors of the values listed in Table 1.1 and 1.2. nd = not determined.

Discussion

Downstream distribution of organic C and N

Research on this semi-natural lotic alpine ecosystem, conducted over the last five years, has revealed that large terrestrial organic material (e.g., woody debris) and DOM are the two major reservoirs of organic C and N. Tight coupling of the terrestrial and aquatic systems along the Tagliamento River promotes high input of plant biomass to the aquatic system (Gurnell et al. 2000; Nat van der et al. 2002). This biomass is either transported by river surface flow or retained by complex floodplain habitat structures (Gurnell et al. 2000).

Woody debris and leaf litter sampled from the land-water interface and surface water showed large seasonal variations in POC concentrations. This variation is probably
resulting from organic material (i) originating from different locations (catchment vs. reach-scale), (ii) having different vascular plant sources, and (iii) showing various degrees of diagenesis (Table 1.1). Small differences in chemical compositions, as resolved from $^{13}$C NMR (Fig. 1.5 B, D), support the conclusion that material is diagenetically ‘young’ or decomposition is slow. Rates of bulk wood decomposition under aquatic under terrestrial conditions along the Tagliamento River (Nat van der et al. 2002) were similar to other literature data (Melillo et al. 1983), but they may underestimate the actual rate of decomposition of large pieces of wood material. In addition, accumulation of wood material with floodplain complexity (Gurnell et al. 2000) involves longer residence times (high retention) in river systems (Robinson 1997). For this reason, we propose that woody material transported by surface water or retained in the system is aged and microbial degradation is slow, because it is hampered by large particle sizes (low surface:volume ratio), low temperatures, and low SRP concentrations. Highest POC and PON concentrations of woody debris in spring and winter may result from long-time deposition during cold periods with low-flow, and concomitant microbial reworking. Whereas lower concentrations in summer and fall may result from rapid removal of reactive or labile components from freshly produced plant biomass.

Figure 1.5
Solid-state $^{13}$C NMR spectra of POM and DOM samples: (A) Benthic algae from R4MC, (B) Salix eleagnos (living wood and leaf biomass) from R4MC, (C) woody debris from R4MC, (D) solid phase
extracted DOM from R4MC, March 1999, (E) solid phase extracted DOM from R4MC, April 1999, and (F) ultrafiltered DOM from R4MC, April 2000. Contaminant peak (*) at ~33 ppm attributed to C18 contamination during solid phase extraction, and (**) at 0 ppm attributed to methyl siloxanes.

PON was selectively removed from woody debris during summer and fall months. Utilization of labile organic N is known to be fast, usually within the order of days, from decomposing woody material (Kaushik and Hynes 1971). Decreasing PON concentrations, therefore, indicate a rapidly cycling component of PON in wood material. On the contrary, abundant concentrations in spring may be explained by an accumulation of refractory and N-rich plant litter during winter months. Enrichment in N is a common phenomenon observed during long-term degradation of organic plant matter (Fahey 1983; Rice 1982; Hedges et al. 1986b).

Benthic algae biomass was moderate to low in the Tagliamento mainstem (but seasonally variable), and primary production may be limited by low SRP concentrations (Arscott et al. 2000). Algal POC and PON concentrations measured in this study were highest in early spring months corresponding to rising temperatures and increased primary productivity stimulated by excess nutrients due to snow melt.

Paucity of measurements of soil sediments (FSPOM) allow for only a limited interpretation of soil-derived POC and PON concentrations. Nevertheless, the high number of land-water interfaces (highly braided system) in this complex river ecosystem suggests high influx of soil biomass to surface waters. Other studies also contend that riverine organic matter is primarily derived from soils (Meybeck 1982; Hedges et al. 1986b). Potentially soil-derived aliphatic/fatty acid material identified in DOM isolates from the Tagliamento waters, further supports leaching of soil microbial biomass into river waters as a source of organic matter (Kaiser et al. 2003a).

Within this study, the abundance of FPOM was low relative to stream size (Meybeck 1981), possibly reflecting a reactive nature. Only ~5% of river-borne TOC is present in form of small particulates and >90% resides in the dissolved phase (Table 2, 3). FPOM abundance was lowest in the pool site, mostly because standing water promotes sedimentation and upwelling groundwater additionally dilutes sestonic concentrations. However, monthly measurements by Arscott et al. (2000) were able to
collect samples associated with flooding peakflows. From their data, particulate C exported during these events could account for nearly 44% of the total C exported. As supported by other studies, the N content of FPOM was high (Malcolm and Duurum 1976; Hedges et al. 1994), and larger longitudinal fluctuations suggest either high biological reactivity or continuous input of FPOM along the river (Table 1.2). These results contradict the finding that POM forms an aged and recalitrant compartment of riverine organic matter (Raymond and Bauer 2001). Kaiser et al. (2003b) observed negligible amounts of clay minerals in this river, therefore, the presence of organic N should not be impacted by interaction with these negatively charged particles. PON concentrations also increased point-wise during post-flood conditions in August 1999. Stepanauskas et al. (2000) observed a strong increase in bioavailable DON concentrations during flooding in a boreal stream. Comparison with their study implies that in the Tagliamento River, larger amounts of labile N (contained in FPOM) may have been mobilized during high discharge. Because FPOM removal is fast, we propose that PON is a rapidly cycling component of riverine organic biomass and partially responsible for the flow of energy in riverine surface waters. Its decomposition heavily determines the relative abundance of dissolved organic and inorganic N (see later C:N).

DOM is the second largest organic C and N reservoir in the Tagliamento River. However, surface water DOC concentrations were low suggesting a reactive chemical nature (Table 1.3). Small quantities of organic substances, which could be isolated, make this system only comparable to other highly oligotrophic surface waters (Benner et al., 1997). Interestingly, the C contents of isolated DOM (cDOC) are comparable to literature values (Thurman, 1986). Variations in DOC and cDOC concentrations partially reflect fluctuations in discharge (Mantoura and Woodward 1983) (Table 3) and seasonal pattern of DOM processing (Table 1.4). It appears as if C gets immobilized during cold periods and low-flow, and released with increasing temperature (a surrogate for biological activity) and water flow. Like in other river systems (Kaplan et al. 1980), in spring, DOM may originate from autochthonous algal and bacterial biomass, which production is stimulated by rising temperatures and increased nutrient supply due to snow melt. In the Tagliamento River, particularly to the major floodplain, high-flow may add an additional amount of DOM originating from decomposition of ‘young’ plant biomass. During
summer and fall low-flow, autochthonous biomass production may decline, but decomposition of terrestrial-derived POM to DOM continues. In winter, although transport and decomposition of POM to DOM is further reduced, accumulated materials age.

Measurements in August 2001 showed that DON concentrations were unusually high relative to DOC (Fig. 1.2 C, D) and, thereby, like for FPOM, support that bulk DOM is highly reactive and cycles fast. The cDON in DOM isolates could not match these very high abundances in bulk DOM. Also, the highly degraded state reflected by NMR experiments (Fig. 1.5 D, E, F), low bacterial utilization and high turnover times of DOM isolates (Kaiser et al. 2003b) are indicative for a recalcitrant character and inefficient downstream processing rates. Therefore, high DON concentrations relate only to high PON in FPOM. We assume that rapid degradation of FPOM releases N-rich DOM of variable molecular weight. Regarding the lower cDON concentrations in the DOM isolates, the dominant portion of DON released must be of lower molecular size and with hydrophilic character. Due to PON chemical composition (in FPOM) and bioavailability the amount and quality of the released DON may vary throughout the year as partly indicated by higher abundances of cDON in the Fall. Then, decomposition processes probably release refractory DON of higher molecular weight, which exhibits decreased turnover rates and, therefore, tends to temporally accumulate (Table 1.4). Kaiser et al. (2003a) identified abundant protein/peptide material in ultrafiltered DOM, which escapes microbial degradation. The unusual biorecalcitrant nature of this proteinaceous material, may also explain DON accumulation. Partitioning of DOM with clay minerals does not impact DON reactivity (see FPOM).

**C:N ratios and the dependency of DOM on POM**

In this study the relationship between C and N is used as an indicator for the reactivity and the relative diagenetic state of the organic matter present in the Tagliamento river system. Woody debris shows the highest C:N ratios. The ratios are similar to those measured from living wood material dominating the riparian vegetation (Karrenberg et al. 2002), and increase from early summer to winter/spring. Very high indicate C-rich plant biomass, being either freshly produced or aged (see above). In contrast, POM with ratios
lower than those from living wood seems to be freshly produced and loss of labile or bioavailable C is rapid (relative to N) (Table 1.1). While wood decomposition, C sequestration may occur fast within initial phases and then continues at lower rate or efficiency, as measured from wood degradation (Melillo et al. 1983).

C:N values of benthic algae (Table 1) indicate that loss of labile C (such as in sugars) may be faster than N removal (Hedges et al. 1988; Cowie and Hedges 1984b) and may explain the high abundance of N (Table 1.1). Selective removal of N in Summer, specifically in the isolated pool, and accumulation during cold periods, supports variable bioavailability and turnover of algae biomass throughout the year. Overall low C:N of algal biomass reflect a reactive nature.

FSPOM also exhibits fairly low C:N ratios (Table 1.1) compared to the literature (Meybeck 1981; Hedges et al. 1986). Soil-derived POM was usually shown to contain refractory humic material with high C:N (~300) (Stevenson et al., 1994), diluted with much lower C:N (8) from microbial biomass (Meybeck 1981). Hedges et al. (1986) observed N-rich fine suspended particles, due to immobilization of N while decomposition of their source materials in soils. As discussed before, N-immobilization is commonly occurring during the degradation of vascular plant tissues in terrestrial (Fahey 1983) and aquatic environments (Rice 1982). Regarding these findings, we suggest that FSPOM from the Tagliamento River may contain a mixture of compounds exhibiting highly variable chemical character, reactivity, and age.

Lowest C:N ratios are found for FPOM, and FPON could account for almost 50% of the biomass (Table 1.2). Measured ratios (3 – 5) are far lower than those (8 – 10) reported in other studies (Meybeck 1981; Hedges et al. 1986). Low ratios and low abundances (see above) of FPOM suggest a highly reactive nature. Selective accumulation of N relative to C from March to November, however, reflects the presence of refractory organic N. Therefore, FPOM composition and reactivity heavily depend on its origin, and microbial decomposition may create specific pattern in N occurrence. Aggregation of dissolved organic compounds appears to be negligible for FPOM formation (Kaiser et al. 2003b).

C:N ratios of DOM isolates (Table 1.3) are comparable to literature values (Thurman, 1986). Due to different fractionation techniques, ratios for solid phase
extracted material were higher than for ultrafiltered isolates (Kaiser et al. 2003a). Since DOM is the second largest energy reservoir in the Tagliamento River, its chemical as well as biological reactivity is of importance. The average ratio calculated from cDOC and cDON is 65, however, from bulk DOC and DON it is 7, which falls at the lower end of values (8 – 41) reported for freshwater DOM (Meybeck 1981; Hedges et al. 1994; Buffam et al. 2001; Tockner et al. 2002). Using both values we can estimate that the largest portion of DOM (permeates) (Kaiser et al. 2003b), which cannot be concentrated by conventional techniques, exhibits an average C:N of 4. The obvious deviation in ratios between DOM isolates and the permeates suggests a large difference in between their chemical compositions. The higher C:N of isolated DOM correspond to ratios measured from FSPOM, benthic algae, and also woody debris, whereas the lower C:N for bulk DOM and the permeates correspond to ratios measured from FPOM. These isolates and permeates also exhibit different bioavailability (Kaiser et al. 2003b). Solid phase extracted and ultrafiltered DOM support low rates of bacterial biomass production and the permeates dominantly foster bacterioplankton growth in Tagliamento surface waters. Thus, DOM leaching from soils or being released by degradation of FSPOM, benthic algae, wood and leaf materials, contributes to the pool of biorecalcitrant and slowly cycling DOM (Kaiser et al. 2003b). The largest portion of DOM is, however, comprised of rapidly cycling and reactive organic compounds, which seem to be derived from FPOM decomposition. Low decomposition rates of wood (years) compared to FPOM (days) further imply that not only the turnover, but also the replenishment of aged and biorecalcitrant DOM is slow.

**C and N isotopic compositions of POM, and sources of FSPOM and FPOM**

δ\(^13\)C values (Table 1.1) and guaiacyl lignin identified by \(^13\)C NMR (Fig. 1.5 B, C) in woody debris point to C\(_3\)-plant origin, confirming their dominant abundance in the riparian and terrestrial vegetation along the river (Karrenberg et al. 2002). Although large POM becomes enriched in \(^13\)C with ongoing decomposition, δ\(^13\)C values are lower than those reported from the literature (Hedges et al. 1986b; Nadelhoffer and Fry 1988). In April, aged woody debris deviated for ~1‰ from living material (Fig. 1.2), and from March to November 1999, C fractionation accounted for up to ~7‰. \(^13\)C NMR spectra of
fresh versus aged wood suggest that while degradation the chemical composition does not significantly change, and hence decomposition of wood appears to be slow compared to other POM sources in the Tagliamento River (Fig. 1.3 B, C). Living wood contains more intact lignin than woody debris, expressed by the shoulder peak at 97 ppm (Fig. 1.5 B) and slightly better resolved aromatic lignin peaks. Interestingly, δ¹⁵N values of woody debris confirm C₃-plant origin, but like δ¹³C are lower than those reported in other studies (Fry 1991; Zah et al. 2001) (Table 1.1, Fig. 1.2). Isotopic ratios were more variable than for C, indicative for higher reactivity.

δ¹³C values of benthic algae are very low and at the lower end of values reported from streams (Fry and Sherr 1984; Hedges et al. 1986b; France 1995; Finlay et al. 1999; Zah et al. 2001). The values do not show any spatial-temporal trends, but reflect generally low primary productivity (Finlay et al. 1999), caused by low SRP in Tagliamento surface waters (Arscott et al., 2000). Benthic algae do not appear to be C limited in the Tagliamento. Benthic algae were rich in ¹⁵N, although heavily depleted in ¹³C. Algae usually exhibit the isotopic signature of their inorganic N sources, especially when inorganic N concentrations are high and non-limiting like in the Tagliamento mainstem (Fogel and Cifuentes, 1993). Thus, N isotopic signatures result from the uptake of heavier nitrate, which is the dominant inorganic N source in this river.

In this lotic ecosystem, FSPOM and FPOM are the two POM compartments, whose sources materials are ambiguous. Both are isotopically heavy, with values corresponding to literature data (Fry and Sherr 1984; Hedges et al. 1986b). These references, e.g., suggest the presence of highly aged plant biomass (humus) and microbial components in FSPOM. A more recent study could identify abundant protein material and potentially soil-derived bacterial fatty acids in DOM isolated from Tagliamento river water (Kaiser et al. 2003a). Leaching of soil biomass was one of the possible explanations for their presence in surface DOM, and suggests that prokaryotic biomass is abundant in soils. Lower C:N from this study, provide additional evidence for dilution of high C:N of degraded plant matter by low C:N of bacteria (4 – 5) (Meybeck, 1981). In contrast, FPOM appears to summarize compounds partially different to FSPOM. Especially due to the high δ¹³C values, we assume that FPOM may derive from FSPOM (Fig. 1.2), as also suggested in other studies (Rounick and Winterbourn 1986; Schiff et
Regarding C:N ratios lower than from FSPOM, we conclude that also autochthonous bacterial and algae biomass contributes to the formation FPOM, however, in small amounts as suggested for watersheds < 10 km² (Finlay 2001). The contribution of algal biomass low in $^{13}$C does not seem to impact the isotopic signature of FPOM, likely due to C fractionation and $^{13}$C enrichment that also occurs during degradation of algal material (Nadelhoffer and Fry 1988). An aggregation of DOM is highly unlikely (Kaiser et al. 2003b). The similarity in $\delta^{13}$C of FSPOM and FPOM is reflected by a similarity in $\delta^{15}$N values (Table 1.1, Fig. 1.2). Common C and N isotopic signatures, thereby suggest that a portion of FPOM derives from FSPOM (see above).

### C and N isotopic compositions and sources of DOM

$\delta^{13}$C values of both DOM isolates compare well with literature values (Hedges et al. 1994; Schiff et al. 1997; Raymond and Bauer 2001). Like C:N ratios, the $\delta^{13}$C of solid phase extracted and ultrafiltered materials strongly differed (by almost 10‰), which may be again explained by the different isolation techniques (Table 1.4). Seasonal isotopic fractionations are likewise in the range of 1 - 2‰ (Fig. 1.4). $^{13}$C NMR also support different chemical compositions (Fig. 1.5 D, E versus F).

Due to these large deviations, we propose partially different origins for solid phase extracted and ultrafiltered isolates. The much heavier $\delta^{13}$C of solid phase extracted DOM are matching with FSPOM, FPOM, and benthic algae. In regard to higher C:N, high aliphatic C, carbohydrate/alcohol, and relatively low aromatic C contents (Fig. 1.5 D, E), we can narrow the selection to FSPOM and benthic algae. Because the abundance of anomeric C from carbohydrates was low in solid phase extracted DOM compared to benthic algae (Fig. 1.5 A versus D, E), algal biomass must have been heavily degraded before entering the DOM pool. FPOM decomposition may may contribute to DOM formation, however, selectively compounds that originate from soils, then leach to surface waters and there add to FPOM biomass. The lower $\delta^{13}$C of ultrafiltered DOM plot closest to those from vascular plant and algae material (Fig. 1.4). Plant material may certainly contribute to the lower $\delta^{13}$C, as indicated by the appearance of phenolic signals (representative of lignin) in the aromatics (Fig. 1.5 B, C). Contribution of algae biomass is relatively low indicated by the low abundance of anomeric C from carbohydrates in
ultrafiltered DOM (Fig. 1.5 A versus F). Although $\delta^{13}$C of bacterial biomass have been shown to widely range between -20 to -50‰ (Cifuentes and Salata, 2001), in light of lower C:N, considerably greater aromatic C and low aliphatic C contents, some amino group carbons (Fig. 1.5 F), and an abundance of protein material identified by solution-state NMR (Kaiser et al. 2003a), we rather suggest soil-derived microbial biomass and autochthonous prokaryotic and eukaryotic biomass origin. Because ultrafiltered DOM was also found to be highly biorecalcitrant to bacterial utilization, their higher protein content indicated by solution-state NMR experiments (Kaiser et al. 2003a) must have been diagenetically altered. The fact, that degradation of organic material generally enriches the C isotopic signature (Nadelhoffer and Fry 1988), makes it hard to explain, which process may lead to a depletion of $^{13}$C in source molecules of ultrafiltered DOM. Possibly C sequestration, due to microbial reworking of FSPOM and FPOM, causes a depletion of the heavy isotope in the resulting DOM fragments.

Interestingly, the average $\delta^{15}$N values of both DOM isolates are identical and plot close to benthic algae (Fig. 1.4), whereas FSPOM and FPOM, identified as important pools of source compounds, are heavier in $^{15}$N. Normally, $^{15}$N is said to accumulate in tropic levels within the food chain (Fry 1991). In river water, bacterial degradation of FSPOM and FPOM may cause N sequestration like for C, explaining the lower $\delta^{15}$N in DOM isolates (Macko and Estep 1983). Similar differences in the isotopic signature of POM and ultrafiltered DOM had been observed for marine samples (Benner et al. 1997). The $\delta^{15}$N values of cDON are less variable than those for FPON, suggesting that the the cDON pool is less dynamic than the small PON reservoir.

We were not able to measure stable C and N isotopes of DOM permeates. But, as discussed before, their low C:N and high bioavailability to riverine bacterioplankton suggest FPOM origin. It seems that FPOM degradation contributes only little to DOM isolates and dominantly releases DOM of smaller molecular weight and high bioavailability. Downstream processing was shown to foster POM decomposition (Table 1.2), and therefore increases the relative amounts of reactive DON in surface waters (Fig. 1.2 D).
**Coupling of organic and inorganic C and N pools**

Input and downstream transformations of organic C and N contribute to pattern of inorganic nutrient concentrations in the Tagliamento mainstem (Fig. 1.2). Therefore, the organic matter chemical composition is crucial for nutrient remineralization. Especially reactive components in POM are responsible for replenishing dissolved organic C and N and inorganic N pools. Pattern created solely by organic matter decomposition in the mainstem are certainly overlayed by input of inorganic nutrients.

FPOM and algae biomass were observed to cycle fast and release reactive DON. Mass balance considerations require additional intense remineralization of inorganic N, such as nitrate and ammonium. Increasing downstream nitrate concentrations also reflect inclining FPOM alteration during longitudinal transport. The overlap of nitrate with SRP and PP concentrations (Fig. 1.2 B, D, E) provide additional evidence for release of inorganic nutrients due to particle decomposition.

If we compare organic to inorganic pools, we find that TOC concentrations are generally low compared to N and P. Average (±SE) TOC:TDN are 2 ± 0.2 and average TOC:TDP are 17 ± 2. These ratios fall far below the expected Redfield trajectories (Redfield, 1958). In contrast, average TDN: TDP are 758 ± 64 high. These inverse relationships suggest intense C- and P-limitation for the microbial community in the Tagliamento mainstem. Indeed, low microbial activity was detected in Tagliamento surface waters (Kaiser et al. 2003c). These nutritional limitations are not changed by point-size nutrient input (Fig. 1.2), because turnover, dilution, or removal of excess C or P is rapid (Fig. 1.2 B, D).

Based on TOC, TDN, and TDP concentrations and average discharge (estimated from measurements between 1994 to 1997) and discharge area, we calculated exports of ~3184 kg C km⁻² y⁻¹, ~632 kg N km⁻² y⁻¹, and ~22 kg P km⁻² y⁻¹ from Tagliamento surface waters to the river estuary and possibly to the northern Adriatic Sea. In comparison, Meybeck (1981) estimated exports of ~3899 kg C km⁻² y⁻¹, but only ~140 kg N km⁻² y⁻¹ and ~11 kg TDP km⁻² y⁻¹. In the Tagliamento River, TOC export is mainly based on POC, which dramatically increased during floods. POC was ~2613 kg C km⁻² y⁻¹ while DOC export was only ~571 kg C km⁻² y⁻¹. From TDN, export of dissolved inorganic N dominated, whereas PN was very low compared to POC. From TDP, PP export
dominated compared to SRP. Overall, it appears as if this river strongly recycles C, however, transports N and P.

Estimates of global riverine C transport to the worlds’ oceans range $\sim0.4 \times 10^{15}$ g C y$^{-1}$ (Berner 1989; Schlesinger and Melack 1981; Meybeck 1981; Hedges et al. 1997). Export of TOC from the Tagliamento mainstem to the river estuary and further to the northern Adriatic Sea accounts $\sim8.2 \times 10^9$ g C y$^{-1}$, which is $\sim10^6$-times less than calculated for the Tagliamento River. As stated above, C export from the Tagliamento is not comparable to other streams of that size (Meybeck 1981). Moreover, retention of large organic material is high in this river system (Gurnell et al., 2000), and although decomposition is slow, reworking of organic matter is strong. If we assume that many rivers were natural before far-reaching human impacts started (>300 y ago) and showed heterogeneity similar to the Tagliamento River, then global C export to the oceans must have been much smaller than nowadays. The dominant portion of riverine organic matter must have been reworked and recycled during its transport to the Sea. Kaiser et al. (2003b) also proposed that small and highly diagenetically altered moieties are dominantly exported to the northern Adriatic Sea, and contribute to the large pool of aged, highly degraded, and lower molecular weight marine DOM. The residence time of higher molecular weight terrestrial organic matter in the oceans is $<130$ y (Opsahl and Benner 1997). Terrestrial-derived DOM of lower molecular weight, which may survive in marine waters for longer times, still escapes chemical characterization due to a high abundance of salts. For this reason, we are not able to establish any global budgets for riverine C export prior to human impact. Further, we are not able to explain, how the processing of C functioned in natural rivers. Therefore, transformations and transport in the natural Tagliamento River may help to better understand riverine C flow over geological time-scales. Many puzzles still need to be solved about the history of the global C cycle, but rivers are key, because the link the C cycle of the continents to the oceans.
References


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Table 1.1
Sample descriptions, concentrations and stable isotope composition of C and N in particulate organic matter (POM) from the River Tagliamento. Samples were taken in March, May, July, August, October, and November 1999, and April 2000. nd = not determined, FSPOM = fine POM from sediment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Collection date</th>
<th>Location *</th>
<th>Wt. %C</th>
<th>Wt. %N</th>
<th>POC (μmol mg⁻¹)</th>
<th>PON (μmol mg⁻¹)</th>
<th>C/N</th>
<th>δ¹³C (%)</th>
<th>δ¹⁵N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>woody debris</td>
<td>March</td>
<td>R4MC</td>
<td>42.6</td>
<td>0.39</td>
<td>270.1</td>
<td>2.11</td>
<td>128.1</td>
<td>-28.2</td>
<td>-6.7</td>
</tr>
<tr>
<td>woody debris</td>
<td>March</td>
<td>R5MC</td>
<td>44.4</td>
<td>0.85</td>
<td>203.4</td>
<td>3.34</td>
<td>60.8</td>
<td>-27.6</td>
<td>-5.4</td>
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<td>May</td>
<td>R2MC</td>
<td>29.5</td>
<td>0.42</td>
<td>161.3</td>
<td>1.96</td>
<td>82.4</td>
<td>-29.1</td>
<td>-8.8</td>
</tr>
<tr>
<td>woody debris</td>
<td>May</td>
<td>R4MC</td>
<td>31.1</td>
<td>0.20</td>
<td>33.7</td>
<td>0.18</td>
<td>185.8</td>
<td>-28.2</td>
<td>-8.6</td>
</tr>
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<td>leaf litter, wood</td>
<td>May</td>
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<td>35.8</td>
<td>0.19</td>
<td>51.3</td>
<td>0.23</td>
<td>224.0</td>
<td>-27.5</td>
<td>-10.7</td>
</tr>
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<td>branches, leaves</td>
<td>July</td>
<td>R2MC</td>
<td>41.6</td>
<td>0.26</td>
<td>34.6</td>
<td>0.19</td>
<td>187.0</td>
<td>-28.3</td>
<td>-6.6</td>
</tr>
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<td>30.2</td>
<td>0.33</td>
<td>91.4</td>
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<td>-1.4</td>
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<td>fresh leaves</td>
<td>July</td>
<td>R4MC</td>
<td>45.0</td>
<td>0.49</td>
<td>43.9</td>
<td>0.41</td>
<td>107.2</td>
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<td>-5.8</td>
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<td>128.0</td>
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<td>0.39</td>
<td>99.2</td>
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<td>44.6</td>
<td>0.41</td>
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<td>0.37</td>
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<td>122.8</td>
<td>-24.1</td>
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<td>0.17</td>
<td>53.8</td>
<td>0.28</td>
<td>190.9</td>
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<td>-7.3</td>
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<td>R4MC</td>
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<td>1.07</td>
<td>61.4</td>
<td>1.25</td>
<td>49.2</td>
<td>-32.5</td>
<td>-3.2</td>
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<tr>
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<td>R4MC</td>
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<td>37.2</td>
<td>0.26</td>
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<td>R4P</td>
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<td>0.19</td>
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<td>101.4</td>
<td>0.35</td>
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<td>0.26</td>
<td>72.7</td>
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<td>-4.2</td>
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<td>0.27</td>
<td>43.3</td>
<td>0.33</td>
<td>132.2</td>
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<td>62.7</td>
<td>0.18</td>
<td>357.3</td>
<td>-34.1</td>
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</table>

*Populus nigra*  
April | R4 | 46.6 | 0.29 | 72.3 | 0.39 | 185.5 | -32.9 | -7.8 |

*Alnus incana*  
April | R4 | 48.6 | 0.55 | 71.7 | 0.69 | 103.2 | -35.1 | -3.3 |

*Salix elegans*  
April | R4 | 45.9 | 0.43 | 82.9 | 0.67 | 123.4 | -35.2 | -4.5 |

*benthic algae*  
March | R4MC | 15.4 | 1.96 | 60.5 | 6.61 | 9.2 | -28.5 | 0.1 |

March | R5MC | 37.5 | 2.08 | 153.7 | 7.29 | 21.1 | -29.7 | 0.1 |

March | R5MC | 8.6 | 0.36 | 31.1 | 1.12 | 27.9 | -35.7 | -2.4 |

March | R5MC | 16.4 | 1.50 | 68.8 | 5.41 | 12.7 | -29.1 | 0.2 |

May | R2MC | 26.9 | 1.14 | 29.2 | 1.06 | 27.7 | -40.3 | -7.9 |

May | R4P | 26.2 | 0.17 | 38.9 | 0.22 | 179.2 | -28.5 | -8.1 |

May | R5MC | 9.9 | 0.12 | 18.1 | 0.19 | 94.1 | -30.2 | -4.8 |

July | R2MC | 10.5 | 0.28 | 21.1 | 0.48 | 43.5 | -39.0 | -4.8 |

July | R5MC | 3.7 | 0.07 | 5.9 | 0.09 | 63.8 | -30.3 | -4.6 |

April | R2MC | 26.1 | 2.35 | 135.4 | 10.45 | 13.0 | -45.5 | -1.4 |

April | R4MC | 16.7 | 1.25 | 47.4 | 3.03 | 15.6 | -32.3 | -0.4 |

FSPOM  
March | R5MC | 2.6 | 0.03 | 18.9 | 0.21 | 90.0 | -23.0 | -2.4 |

Nov | R4P | 0.3 | 0.01 | 3.3 | 0.12 | 26.9 | -25.2 | -0.3 |

April | R4P | 0.1 | 0.00 | 0.4 | 0.02 | 16.3 | nd | -4.7 |

April | R5MC | 1.4 | 0.04 | 15.3 | 0.36 | 42.6 | -28.0 | -2.4 |
Sample descriptions, %POC recovered, and concentrations and stable isotope compositions of C and N in particulate organic matter filtered onto GF/F filter (>0.7 µm) from the River Tagliamento. %POC recovered was calculated as the percentage of TOC concentrations. Samples were taken in March, May, July, August, October, and November 1999, and April 2000. nd = not determined

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<th>Wt. %C</th>
<th>Wt. %N</th>
<th>POC (µM)</th>
<th>PON (µM)</th>
<th>C/N (atom)</th>
<th>δ^{13}C (‰)</th>
<th>δ^{15}N (‰)</th>
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Table 1.3
Sample descriptions, dissolved organic carbon (DOC) concentrations, and mass balance results for C18-solid phase extraction and ultrafiltration. %DOC recovered from C18-solid phase extraction and ultrafiltration after concentration and freeze-drying. Solid phase extracted = SPE, nd = not determined.

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<th>DOC (µM)</th>
<th>Volume (L)</th>
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<td>SPE</td>
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* R2, headwater floodplain; R4, major floodplain; R5, lower floodplain; MC, main channel; P, isolated pool in major floodplain
Table 1.4
Concentrations (cDOC, cDON) and stable isotope compositions of C and N in solid phase extracted and ultrafiltered dissolved organic matter from the Tagliamento River. See Table 2 for sample descriptions.
Solid phase extracted = SPE, nd = not determined.

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<th>Wt. %N</th>
<th>cDOC (µM)</th>
<th>cDON (µM)</th>
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<td>0.04</td>
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<td>0.15</td>
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* R2, headwater floodplain; R4, major floodplain; R5, lower floodplain; MC, main channel; P, isolated pool in major floodplain
CHAPTER 2

Solid-state and multidimensional solution-state NMR of solid phase extracted and ultrafiltered riverine dissolved organic matter (DOM)

KAISER, E., SIMPSON, A. J., DRIA, K. J., SULZBERGER, B. AND HATCHER, P. G.

In this study we used multidimensional solution-state NMR to elucidate the differences in the chemical composition of solid phase extracted and ultrafiltered DOM isolates. DOM was isolated from water sampled from an oligotrophic river, the River Tagliamento (Italy). The recovery of total DOM was up to 42% with both isolation techniques. In addition to 1- and 2-D solution-state NMR, we also applied 1-D solid-state $^{13}$C NMR spectroscopy for DOM characterization. $^{13}$C NMR spectroscopy only produced broad overlapping resonances, thus allowing a bulk characterization of DOM composition. However, it demonstrated that the bulk chemical composition of the two DOM fractions exhibited minor spatial-temporal changes. The 2-D experiments (TOCSY, HMQC) showed that the solid phase extracted hydrophobic DOM contained predominantly aliphatic esters, ethers, and hydroxyl groups, whereas the ultrafiltered DOM was comprised partially of peptides/protein, with further evidence for a small amount of aliphatic/fatty acid material. Sugars were present in both DOM fractions. The results show that the two isolation techniques selected for different suites of compounds within the bulk DOM pool.
**Introduction**

Multiple analysis techniques, ranging from NMR spectroscopy to GC-MS, have been applied to examine the specific chemical features of DOM (Hedges et al. 1992; Buddrus et al. 1989; Lampert et al. 1992; Hatcher and Clifford 1994). Most of these techniques require that DOM, present in low concentrations in natural water samples, be concentrated using isolation methods such as XAD- or C18-solid phase extraction and ultrafiltration (Stevenson 1994). These methods also allow recovering DOM as a solid powder that can be analyzed by solid-state NMR (Aiken et al. 1985; Louchouarn et al. 2000). Hitherto, mostly one-dimensional (1-D) NMR spectroscopy, solid-state $^{13}$C NMR in particular, was applied to elucidate the DOM chemical composition. However, many published solid-state $^{13}$C NMR spectra exhibit only broad resonance peaks, often suffer from poor spectral quality due to sample complexity, and some even contain “spinning side bands”. Advances in solid-state NMR have drastically reduced the effect of spinning side bands and allow the analysis of smaller sample quantities with increased sensitivity (Dria et al. 2002). Nevertheless, these NMR spectra still often exhibit broad overlapping peaks, due to the presence of paramagnetic materials and the dipolar interactions present in solid materials. In contrast, multidimensional solution-state NMR can give highly resolved information, even for complex mixtures (Simpson 2001). Multidimensional experiments (Croasmun et al. 1994) can be used to correlate the chemical shifts of different carbon and proton atoms and identify short- and long-range connectivities.

In using isolation techniques to concentrate DOM, one must be aware that different isolation methods, e.g., C18-solid phase extraction and ultrafiltration, select for chemically different compounds. This is the first study in which homo- and multi-nuclear solution-state NMR methods were used to compare the chemical composition of DOM isolated by C18-solid phase extraction and ultrafiltration. The goals of our study were (i) to use state-of-the-art NMR methods to elucidate the differences in the chemical composition of DOM isolates derived by chemical and physical fractionation techniques, and (ii) to demonstrate that solution-state NMR spectroscopy is a tool of high potential for investigating the molecular nature of heterogeneous DOM mixtures. We show that NMR methods improve functional group assignments and aid in the understanding of DOM chemical structure.
Material and Methods

Study area and sample collection
C18-solid phase extracted hydrophobic and ultrafiltered DOM samples come from water of the Tagliamento River, northeastern Italy. The river is the last unregulated river in Europe, flowing unrestrained from the alpine headwaters to its mouth in the northern Adriatic Sea (Ward et al. 1999).

Water samples for C18-solid phase extraction and ultrafiltration were collected seasonally from the main channel in the headwater, major, and transition floodplains from spring 1999 to 2000. The samples are part of a larger study in which material was collected seasonally from three longitudinally aligned stations (Kaiser et al., in preparation). Water was collected during low-, medium-, and high-flow conditions (Table 2.1). Throughout the year, the main channel river water displayed temperatures of 10.9±2.5°C, pH’s of 8.13±0.14, and alkalinities of 2.8±0.5 mM. Water samples for DOM fractionation were collected with clean 50 L high-density polyethylene (HDPE) carboys and transported back to the field station. Immediately following collection, water samples were filtered through muffled glass fiber filters (GF/F) and pre-rinsed 0.2 µm Durapore filters (142 mm diameter, Millipore), and stored in clean 50 L carboys.

DOM fractionation and isolation
For chemically fractionating DOM into hydrophobic and hydrophilic compounds, a Mega Bond Elute C18 column (C18 loaded silica, 60CC, Varian) was used after acidification with 32% hydrochloric acid (Suprapur) to pH 2.8. Flow rates ranged between 3-5 L h⁻¹. Of the total dissolved organic carbon (DOC <0.2 µm), 29-42% was recovered as a sorbed hydrophobic fraction (Table 2.1), being comparable to other literature values (Louchouarn et al. 2000; Amador et al. 1990). A Filtron tangential flow ultrafiltration system with a polyethersulfone membrane (1 kDa nominal weight cutoff) was used for fractionating bulk DOM into pseudo high- and low-molecular weight DOM portions, following the protocol of Benner et al. (Benner et al. 1997). Filtration rates ranged between 6-8 L h⁻¹ using one 0.46 m² cassette filter (Centrasette, Filtron). The water temperature ranged from 20-22°C during ultrafiltration. Recovery of isolated DOC was 12-22% of the total DOC (Table 2.1).
The total organic carbon (TOC) concentrations of the unfiltered water and the DOC concentrations of all different DOM fractions were measured to determine a carbon mass balance (Table 2.1). Samples were stored at 4°C in the dark and immediately transported to the laboratory (EAWAG, Switzerland). DOM adsorbed on the C18-phase was eluted by gentle vacuum-filtration using HPLC-grade methanol (Merck). The methanol was removed from the DOM by roto-evaporation and freeze-drying. Next, the extract was redissolved in MQ-UV water (Millipore). The aqueous solid phase extracted or ultrafiltered DOM samples were cation-exchanged (Bio-Rad, AG 50W and AG MP-50) to remove trace metals (Everett et al. 1997), and then concentrated by freeze-drying for further elemental and spectroscopic characterization.

Tar-like ultrafiltered DOM (except the April 2000 sample) was redissolved in methanol and freeze-dried. However, we could not reach solid phase or completely remove all the methanol from the ultrafiltrate, as shown by the intense resonance at 55 ppm in the solid-state NMR spectra (Fig. 2.1 E, F, G). Redissolution in MQ-UV water, neutralization to pH 8.1, and subsequent freeze-drying finally resulted in reaching a solid phase. The dried material was scraped from the Teflon-beaker, and stored sealed in muffled glass tubes (Supelco) at 4°C in the dark. Solution-state NMR experiments were performed only on solid phase extracted DOM from early summer 1999 and ultrafiltered DOM from spring 2000, due to sufficient time and sample availability.

**TOC and DOC measurements**

Water samples were collected directly after fractionation for ultrafiltered samples and after freeze-drying and redissolution in MQ-UV water for solid-phase extracted DOM. The samples were collected in acid-rinsed, muffled 40 ml EPA glass vials, sealed with Teflon-lined caps and stored frozen until analysis. TOC and DOC concentrations were determined by high temperature catalytic oxidation with a Shimadzu 5050A analyzer (Benner and Strom 1984).

**Solid-state $^{13}$C ramp NMR spectroscopy**

NMR spectra were obtained as described by Zang et al. (2000) and Dria et al. (2002) using the ramp cross polarization magic angle spinning (ramp CPMAS) pulse program
and two pulse phase modulated (TPPM) decoupling on a Bruker DSX 300 NMR spectrometer, operating at a frequency of 300 MHz for $^1$H or 75.48 MHz for $^{13}$C. Approximately 20-30 mg of sample was placed in a 4 mm (outside diameter) NMR rotor between Teflon spacers and sealed with a Kel-F® cap. Samples were spun at a frequency of 13 kHz using a contact time of 2 msec and a 1 sec recycle delay time. For the samples, 80,000-100,000 acquisitions (scans) were collected. For each sample’s free induction decay, 1,024 complex data points were collected and zero-filled to a total of 4,096 data points. The samples were Fourier-transformed, and 100 Hz line-broadening was applied and phased appropriately. The carboxyl carbon of glycine (176.03 ppm) provided a secondary reference for all solid-state spectra.

**Solution-state NMR spectroscopy**

Before analysis by NMR spectroscopy, samples were resuspended in MQ-UV water and, to ensure solubility, the pH raised to 10 using a 0.1 M NaOH solution. To remove excess metal ions, the DOM solution was passed in series over two IR-1200H cation-exchange resins. The eluent was freeze-dried and further dried over P$_2$O$_5$ at 35°C for 24 hours to reduce the water signal in the proton NMR spectra. Finally, the DOM (~20 mg) was dissolved in DMSO-d$_6$ (0.75 ml). Solution-state NMR data were acquired using a Bruker Avance 400 MHz NMR spectrometer fitted with a QNP $^1$H, $^{13}$C, $^{15}$N, and $^{31}$P probe. 1-D proton NMR (1,024 scans) analyses were carried out with a 2 sec recycle delay time and processed with 1 Hz line-broadening. Total Correlation Spectroscopy (TOCSY) spectra (256 scans, TD (F1) 1,024, TD (F2) 512) were acquired using a 60 msec mixing time, with Time-Proportional Phase Incrementation (TPPI). The data processing used a sine-squared function with a phase shift of 90° in both dimensions. Heteronuclear Multiple Quantum Coherence (HMQC) spectra (512 scans, TD (F1) 1,024, TD (F2) 512, J$_1$ ($^1$H-$^{13}$C) 145Hz) were acquired using a Bilinear Rotation Decoupling (BIRD) pulse train and TPPI. F1 was obtained with a sine-squared function with phase shift of 90°, while F2 was processed with a Gaussian-broadening of 0.001 and line-broadening of –1.
Results and Discussion

Bulk chemical characterization of solid phase extracted and ultrafiltered DOM

Generally, DOC concentrations fluctuate with discharge (Mantoura and Woodward 1983), exhibiting low levels at low flow and vice versa, indicating that DOC concentrations reflect well river hydrodynamics. In this study, the amount of surface water DOM retained by solid phase extraction and ultrafiltration did not correlate with total DOM (Table 2.1).

Solid-state $^{13}$C NMR spectra of DOM fractions extracted by the two techniques exhibit a clear difference (Fig. 2.1 A-D versus E-H), especially when comparing DOM fractions from spring 2000 in which the DOM isolates were from the same water sample (Fig. 2.1 D, H). Due to time and sample limitations, solution-state NMR studies could be only performed on a limited number of samples. However, the solid-state NMR spectra (Fig. 2.1) as well as additional spectra presented elsewhere by Kaiser et al. (in preparation) showed that the chemical composition of DOM did not significantly change spatially or temporally.

Table 2.1
Sample descriptions, dissolved organic carbon (DOC) concentrations, and mass-balance results for ultrafiltration and C18-solid phase extraction. CF (concentration factor) = ratio of the initial sample volume to the volume of retentate (solid concentrate) at the end of ultrafiltration (solid phase extraction). % aliphatic = 0-110 ppm and % aromatic = 110-160 ppm from solid-state $^{13}$C NMR analyses after contaminant subtractions.

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<th>Location*</th>
<th>DOC (µM)</th>
<th>Volume (L)</th>
<th>DOM fraction</th>
<th>C F</th>
<th>% DOC retained</th>
<th>% aliphatic</th>
<th>% aromatic</th>
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* R2, headwater floodplain, R4, major floodplain, R5, transition floodplain; MC, main channel.
One-dimensional (1-D) solid-state NMR

The 1-D solid-state ramp $^{13}$C NMR spectra of DOM isolated by both methods (Fig. 2.1) show broad resonances. Some of the spectra exhibit some sharp peaks that are uncharacteristic of similar published spectra of DOM and may be caused by contaminants (artifacts). The sharp-clipped peaks at 33 ppm observed in the solid phase extracted DOM spectra (Fig. 2.1 A-D) may be from bleed of the C18 phase. However, these sharp peaks are narrow and likely constitute <2% of the sampled carbon. Ultrafiltered DOM spectra E, F, and G show intense peaks at 56 ppm not observed in solid phase extracted materials. These peaks probably resulted from the production of methyl esters during the drying of the methanol extract or may be from residual sorbed methanol. The peak at 0 ppm observed in the spectra of ultrafiltered DOM was probably from methyl siloxanes of undetermined origin.

Spectra obtained by both extraction techniques exhibit four major functional groups of organic compounds: aliphatics, carbons adjacent to O or N (such as in carbohydrates, amino acids, esters, alcohols, etc.), aromatics, and carboxyls-/aliphatic amides. The chemical shift of more defined signals indicate specific functional groups or structures, such as: alkyl groups (12-25 ppm), methylene (29-35 ppm), sugars, aliphatic methine, alcohols, methoxyl and amino carbons (45-90 ppm), possible anomeric sugars (90-110 ppm), aromatics and alkenes (110-140 ppm), aromatic carbon adjacent to oxygen (140-160 ppm), carboxylates, aliphatic amides (160-190 ppm), and carbonyls and ketones (190-230 ppm).

Except for sample spectrum B, little seasonal variability is observed (Fig. 1 A-D, E-H). Any such seasonal variability is certainly less than differences observed between the two extraction techniques. All spectra display the same carboxylic acid/aliphatic amide (160-190 ppm) signal intensities. The solid phase extracted DOM samples contain high aliphatic carbon contents, high carbohydrate/alcohol contents and relatively low aromatic carbon concentrations. Ultrafiltered DOM spectra have considerably greater aromatic carbon contents, lower aliphatic carbon contents and a higher abundance of methoxy/amino group carbons compared with spectra of solid phase extracted DOM. Furthermore, the ultrafiltered samples exhibit minor peaks for carbohydrate/alcohol carbons and appear to have a lower aliphatic carbon content than solid phase extracted
DOM. The carbon resonances for aromatics from ultrafiltered DOM showed phenolic signals, possibly indicating the contribution of lignin.

Fig. 2.1
Solid-state ramp $^{13}$C NMR spectra measured from solid phase extracted DOM sampled from the main channel in (A) spring 1999, (B), early summer 1999, (C), fall 1999, and (D) spring 2000, and from ultrafiltered DOM sampled from the main channel in (E) early summer 1999, (F) summer 1999, (G) fall 1999, and (H) spring 2000 (see also Table 2.1).

**Multidimensional solution-state NMR**
A combination of 1- and 2-D solution-state NMR techniques were applied to the solid phase extracted and ultrafiltered DOM. Conventional 1-D proton NMR spectra were obtained in DMSO-d$_6$ and then reacquired after addition of D$_2$O, allowing identification of exchangeable functionalities that disappeared in the presence of D$_2$O (Simpson et al. 1997; Kingery et al. 2000). We also performed homonuclear Total Correlation
Spectroscopy (TOCSY) and Heteronuclear Multiple Quantum Coherence (HMQC) experiments. TOCSY allows detection of proton bond couplings in an entire spin system and HMQC correlates $^1$H and $^{13}$C chemical shifts (over the range of one H-C bond). For both methods, we observed very short $T_2$ relaxation times, most likely resulting from a combination of variable magnetic susceptibility of the high molecular weight heterogeneous samples, persistence of paramagnetic metals, exchange processes, and rigidity. As a result, the 2-D spectra underestimate the contributing molecules with very short $T_2$ relaxation (broader signals), as these signals decay during the 2-D pulse sequence.

**Multidimensional solution-state NMR of solid phase extracted DOM**

Four major spectral regions can be identified (Fig. 2.2 A): (1) aromatics, (2) broad signal from water, (3) signals from amino acids (protons on $\alpha$-carbons, and various $\beta$, $\gamma$-carbons), sugars, methylene adjacent to ester and ether/hydroxyl groups, and (4) various aliphatic units (Simpson 2001; Simpson et al. 2001; Ede and Kipelainen 1995). Addition of D$_2$O (Fig. 2.2 B) caused the broad water signal (region 2) to shift to a sharper signal (centered around ~3.8 ppm), but otherwise had minimal impact on the spectrum.

Tentative assignments from the TOCSY and HMQC data are given in figure captions 2.3 A, 2.3 B, and 2.4. The TOCSY experiment confirms some of the major assignments (Fig. 2. 3). Strong crosspeaks in the aromatic region of the HMQC (and also observed in the TOCSY data, Fig. 2.3) are consistent with phthalate and phthalic acid. These compounds may originate from storage in HDPE carboys or from natural riverine organic compounds (Simpson et al. 2001). Resonances in the 2-D experiments are consistent with lignin, carbohydrates, and aliphatic esters/acids/ether. Both the $^{13}$C solid-state (Fig. 2.1) and $^1$H solution-state NMR spectra (Fig. 2.2) support the presence of these structures in the solid phase extracted DOM. Lignin-derived methoxy carbons and protons are identified as region 10 in Fig. 2.4.
Fig. 2.2

(A) $^1$H NMR of solid phase extracted DOM solubilized in DMSO-d$_6$: (1) aromatics, (2) water, (3) a mixture of amino acid, sugar, CH$_2$ adjacent to ester and ether/hydroxyl, and (4) various aliphatic units. (B) The $^1$H of the same sample after the addition of a small amount of D$_2$O. * indicates DMSO-d$_6$, ** indicates water (HOD) after the addition of D$_2$O.
Fig. 2.3

(A) Full TOCSY spectra of solid phase extracted DOM sampled from the main channel in early Summer 1999. (B) Expanded region of the TOCSY spectra: (1) methine, methylene couplings in unsaturated hydrocarbon chains, (2) sugar couplings, (3) couplings from protons $\alpha$ to the oxygen of esters to protons in aliphatic chains (-COO-CH$_2$-CH$_2$(CH$_2$)$_n$), (4) couplings from protons $\alpha$ to ethers/alcohols to protons in
aliphatic chains (-O-CH2-(CH2)n-), (5) couplings from protons α to the carbonyl of esters/acids to protons in aliphatic chains (-CH2-(CH2)n-COO-), (6) protons β to ether/hydroxy/ester coupling to protons in aliphatic chains, and (7) main chain methylene in aliphatic chains coupling to terminal or branched methyl groups.

Fig. 2.4
HMQC spectrum of solid phase extracted DOM solubilized in DMSO-d6: (1) aromatic couplings consistent with those on a 1,2 substituted ring, (2) weak sugar couplings, (3) methylene adjacent to ester, CH2-OOC, (4) methylene adjacent to ether or hydroxyl CH2-OR (where R is H or alkyl unit), (5) DMSO (solvent), (6) methylene adjacent to a carboxylic acids/ester (-CH2-COOH/COOR), (7) methylene once removed from a carboxylic acids/ester (-CH2-COCH2-COOH/COOR), (8) main chain methylene in hydrocarbon chains (-CH2-(CH2)n-CH2-), (9) terminal or branched methyl groups, and (10) aromatic methoxy.
Multidimensional solution-state NMR of ultrafiltered DOM

The 1-D $^1$H NMR spectrum of ultrafiltered DOM dissolved in DMSO-$d_6$ is shown in Fig. 2.5 A. Five major regions can be identified: (1) amides, (2) ammonia, (3) predominantly sugars, (4) protons on the $\alpha$-carbon and side-chain carbons of amino acids, and possible contributions from methine/methylene adjacent to aliphatic ester/ether/hydroxyl and methine, (5) methylene units bridging lignin aromatics, and aliphatic structures (including resonances from amino acid side-chains). The addition of D$_2$O reduced the intensity of region 1 (Fig. 2.5 B), suggesting the protons are exchangeable and, therefore, consistent with amides (Simpson et al. 1997; Kingery et al. 2000; Simpson et al. 2001). Moreover, it unmasked resonances for aromatic protons that can now be clearly observed (region 5).

The 2-D TOCSY (Fig. 2.6) and HMQC (Fig. 2.7) spectra support assignments made from the 1-D spectra (Fig. 2.5). The 2-D experiments indicate the presence of peptides, carbohydrates, protons in long-chain aliphatic structures, aromatic protons, and ammonia. It is possible that the aromatic protons are, in fact, part of the peptides, as there is no evidence of methoxy (generally associated with lignin-type aromatics) in the HMQC spectra. Lignin-derived methoxy gives a very characteristic and strong resonance at 3.7 ppm for proton and at 56 ppm for carbon in HMQC-type experiments (Ede and Kipelainen 1995; Ede and Ralph 1996), but it is not present in Fig. 2.7. The signal centered ~56 ppm in the CPMAS spectrum (Fig. 2.1 H) may largely result from $\alpha$-carbons in peptides which also resonate around this chemical shift. The intensity of the amide region in the $^1$H spectrum (Fig. 2.5) indicates a significant presence of protein/peptide-derived matter. Based on the total proton intensity in the sample, an integration of the amide region estimates that ~4% of the total proton intensity can be attributed to the N-H of amides. If we consider the contribution of the associated side-chains in each residue to the overall intensity, then the actual amino acid/peptide contribution to the total $^1$H intensity of the sample may be ~16-32% (note that for every amino acid there will be 4-8 additional protons other than the N-H of amides and dependent on the exact amino acid unit). A C:N ratio of 37 (Kaiser et al., in preparation) further suggests that a mixture containing 20% peptide structures may exhibit a C:N ratio of 30-40, assuming no other source of N. The exact source of the ammonia is unclear, but its resonance is apparent in many soil-derived humic-type materials dissolved in DMSO-
d$_{6}$. It is possible that the ammonia is sorbed/entrapped within the DOM matrix, and therefore may originate from natural sources. However, ammonia also may result from the hydrolysis of labile peptide structures during the sample preparation for solution-state NMR analysis (see methods).

Fig. 2.5
(A) $^1$H NMR of ultrafiltered DOM solubilized in DMSO-$d_{6}$. (B) $^1$H of the same sample after the addition of a small amount of D$_2$O. The major spectral regions have been labeled as (1) amides, (2) ammonia, (3) sugars and methine, (4) protons on the $\alpha$ carbon and on carbons of some amino acid side chains, aliphatic units including some amino acid side chains, and (5) aromatic structures (including resonances from amino acids side chains). * indicates DMSO-$d_6$, ** indicates water.
Fig. 2.6
TOCSY experiment of ultrafiltered DOM sampled from the main channel in spring 2000. (A) Major
couplings were identified as (1) peptides (amide-αβγ couplings), (2) aromatic couplings and couplings from
ammonia, (3) peptides (α-β and β-γ couplings), sugars, and aliphatic structures. (B) Unambiguous
assignments from the TOCSY spectra were not possible, but following Fan et al. (2000) we tentatively
suggest: (1) serine*, (2) various carbohydrate structures, (3) carbohydrates, (4) glutamate*, (5) alanine*, (6)
threonine*, valine* (and possible contributions from additional amino acids), and contributions from COO-
\( \text{CH}_2-\text{CH}_2-\text{CH}_2^- \) in aliphatic-type structures, (7) leucine*, (8) methylene adjacent to ether or hydroxyl \( \text{CH}_2-\text{OR} \) (where R is H or alkyl unit) and possibly contributions from additional amino acids such as histidine*, arginine* and cysteine*, (9) couplings in fatty acids/esters (\( \text{CH}_2-(\text{CH}_2)_n-\text{COOH/COOR} \)), (10) methyl terminating hydrocarbon chains (\( \text{CH}_2-n-\text{CH}_3 \)) such as those in fatty acids or esters (\( \text{RHOOCH}2(\text{CH}_2)_n-\text{CH}_3 \)), and (11) couplings in \( \beta \) hydroxy fatty acids (\( \text{CH}_2-\text{CHOH-CH}_2-\text{COOH} \)).

*Note that the chemical shifts and couplings suggest these residues are present as part of a peptide structure.

**Fig. 2.7**

HMQC spectrum for ultrafiltered DOM shows a range of crosspeaks that are consistent with (1) signals from methylene adjacent to ether hydroxy \( \text{CH}_2-\text{OR} \) (where R is probably H or alkyl unit), (2) various units that are consistent with either alkyl units adjacent to ester, ether or hydroxyl, sugars, or some amino acids, (3) DMSOd\(_6 \) (solvent), (4) methylene adjacent to a range of carboxylic acids/esters (\( \text{CH}_2-\text{COOH/COOR} \)), (5) methylene once removed from a carboxylic acid/ester (\( \text{CH}_2-\text{CH}_2-\text{COOH/COOR} \)), (6) main chain methylene in hydrocarbon chains (\( \text{CH}_2-(\text{CH}_2)_n-\text{CH}_2^- \)), and (7) methyl groups.
**Chemical extraction versus physical fractionation: A comparison**

In general, C18-solid phase extraction separates molecules according to hydrophobicity, and ultrafiltration separates by size and shape. Solid-state $^{13}$C NMR analysis of DOM samples prepared by solid phase extraction show elevated aliphatic:aromatic ratios compared to ultrafiltered samples (Table 2.1). When comparing solid-state NMR spectra of seasonally collected DOM by both techniques, we can resolve large differences in broad resonances and, thus, in the chemical composition of these two types of DOM isolates (Fig. 2.1).

As shown in Figure 1, the coarse resolution of solid-state NMR allows us to discriminate only to a certain extent the different chemical structure of isolated DOM fractions. Solution-state NMR better resolves molecular patterns in DOM structure. Solid phase extracted DOM mainly contains aliphatic esters, ethers, hydroxyl groups, and smaller quantities of sugars. Ultrafiltered DOM is partially composed of peptides and proteins, with further evidence for aliphatic/fatty acid material and sugars. The solid-state $^{13}$C NMR spectra indicate a higher content of aromatic material in this fraction, as compared with the solid phase extracted DOM fraction. It appears as if C18-solid phase extraction preferentially selects for aliphatic compounds and loses important structures such as proteins, aromatics and some sugars. Ultrafiltration recovers a much wider range of biomacromolecules, dominated by proteins, sugars, and fatty acids.

**Environmental significance**

Resolving the molecular traits of freshwater DOM components is key for understanding the sources and processing of organic compounds in riverine systems and their role in the global carbon cycle. Rivers not only transport but also transform organic compounds, and therefore link the carbon cycle of the continents to that of the oceans (Meybeck 1982; Raymond and Bauer 2001).

NMR analysis of solid phase extracted DOM from the River Tagliamento has shown a dominance of aliphatic esters. As bacteria and microalgae constitute an important pool of living biomass in aquatic systems and are major sources for DOM (Hedges et al. 1994; 1997), we suggest that this lipophilic DOM isolate comprises
important microbial membrane components such as those found in prokaryotes and algae. Similar sources were identified for hydrophobic-type DOM extracted from soils (Guggenberger et al. 1994; Conte et al. 1997). The ether and hydroxyl groups identified in the solid phase extracted DOM are associated with aliphatic chains, and hence are probably derived from either transformed plant inputs into the river, such as plant cuticles, or from hydroxy fatty acids, generally indicative of cellular material. We also identified some signals attributed to sugars, which are derived from multiple sources such as cellulose and hemicellulose from vascular plants, algal biomass and microorganisms (Hedges et al. 1992; Aiken et al. 1984; Gilliam at al. 1985). We suggest a predominant vascular plant origin for these sugars because these materials constitute substantial components of riparian vegetation Hedges et al. 1997; Fan et al. 2000), and there is tight coupling between terrestrial and aquatic areas along the Tagliamento river. The presence of sugars indicates that these compounds are either diagenetically reworked or persist protected and therefore escape degradation processes. A recent study by Kaiser et al. (in preparation) showed that the utilization of this solid phase extracted DOM by natural bacterioplankton was very low and reflected its non-reactive nature.

The analysis of ultrafiltered DOM from the River Tagliamento shows the presence of peptide/protein-type compounds. Their occurrence is of special interest because proteins/peptides are usually viewed as labile DOM components with relatively short biological turnover times, ranging on the order of minutes to hours (Fuhrman 1990). However, Kaiser et al. (in preparation) found that this ultrafiltered DOM was not readily consumed by natural bacterioplankton. The protein/peptide structures identified from the ultrafiltered DOM may indicate the presence of degradation-resistant protein structures, possibly bacterial-derived membrane components or carbohydrate cross-linked amides (Tanoue et al. 1995; Ogawa et al. 2001). The formation of microbially resistant ligno-protein complexes in humus has been documented (Waksman 1932). Several recent studies proposed the encapsulation of protein in humic acids (Zang et al. 2000; Knicker and Hatcher 1997). If proteins contained in the ultrafiltered DOM from the River Tagliamento, originated from surrounding soils, they may have undergone similar diagenetic transformations, making them less susceptible to microbial utilization. For future studies, we suggest to investigate the structure and bioavailability of this
protein/peptide-rich material to better understand its origin, transformation, and fate in natural freshwater and marine systems. Furthermore, we emphasize the potential of 2-D NMR studies added to the list of methods that can be utilized in such ventures.

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Literature Cited


CHAPTER 3

Chemical and molecular characteristics and bioreactivity of riverine dissolved organic matter (DOM)

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Downstream and seasonal variability in the chemical and molecular compositions of different riverine DOM fractions were studied and linked to their bioavailability to natural bacterioplankton. Chemical- and size-fractionation (C18-solid phase extraction and ultrafiltration) revealed that riverine DOM consisted of 60 - 80% of hydrophilic-type and low-molecular weight (LMW) and of 20 - 40% of hydrophobic-type and high-molecular weight (HMW) organic compounds. We were able to recover hydrophobic and HMW DOM in a solid phase, which facilitated extensive chemical characterization. Solid-state ramp CPMAS $^{13}$C NMR and TMAH-GC/MS analyses revealed that retained DOM is mostly derived from vascular plant material and soil biomass, and to a lesser extent from autochthonous microbial production in the river. The isolates also showed a high degree of diagenetic alteration and behaved biorecalcitrant to bacterial uptake. In contrast, the bioavailability of hydrophilic and LMW DOM were always highest. Further, size exclusion chromatography (SEC) revealed that bacteria decompose all DOM fractions to compounds of lower molecular weight with potentially reduced bioavailability. On a spatial-temporal scale we observed little changes in DOM chemical composition and bioavailability in the Tagliamento River. Overall, we suggest fast downstream processing of hydrophilic and LMW DOM and transport of aged and degraded hydrophobic and HMW compounds.
Introduction

Organic carbon is essential to all life processes and DOM represents a major reactive reservoir of reduced organic carbon within aquatic systems (Schlesinger and Melack 1981; Meybeck 1981; Hedges et al. 1997). River systems, for example, annually discharge sufficient organic material (approximately $0.4 \times 10^{15} \text{ g C yr}^{-1}$) to account for the global turnover of dissolved organic carbon (DOC) in the vast oceanic realm and for the temporary removal of organic carbon through marine sedimentation (Williams and Druffel 1987; Berner 1989; Hedges and Keil 1995). However, rivers not only transport organic matter, but also alter and solubilize it through biological, chemical, and physical reworking (Ittekot 1988; Hedges et al. 1994; Raymond and Bauer 2001). Therefore, the origin and molecular features of DOM are crucial for its transformations within aquatic ecosystems (Wetzel 1992; Hedges et al. 1992; 1997; Findlay and Sinsabaugh 1999).

The application of different fractionation and isolation techniques has been of great help in composition studies. Solid phase extraction and ultrafiltration have facilitated the concentration of hydrophobic and high-molecular weight (HMW) DOM fractions into solid form (Aiken et al. 1985; Hedges et al. 1992; Opsahl and Benner 1997; Louchouarn et al. 2001). This has allowed detailed chemical characterization by techniques such as one-dimensional solid-state $^{13}$C NMR (Aiken et al. 1985a; Hedges et al. 1997) and multidimensional solution-state $^{13}$C NMR (Buddrus et al. 1989; Lampert et al. 1992) to unravel the complex nature of macromolecular freshwater DOM isolates (Roubeuf et al. 2000; Kaiser et al. 2003a).

The type of information provided by such detailed studies of DOM needs to be related to its bioavailability to the microbial community. Microorganisms are the major consumers of DOM in aquatic systems (Cole et al. 1988) and play a key role in the biogeochemical cycling of DOM in rivers (Findlay et al. 1991; 1992; Leff 1991). It has been documented that HMW DOM in lakes and oceanic surface waters is more rapidly and efficiently utilized by natural bacteria than low-molecular weight (LMW) DOM (Tranvik 1990; Amon and Benner 1996). Other studies report higher bioavailability of LMW DOM (Kaplan et al. 1980; Meyer et al. 1987). Bacterial uptake of bulk DOM and its various chemical- and size-fractions have traditionally been used to assess bioavailability or recalcitrance of DOM (Amon and Benner, 1996). Accordingly, the
potential fates of specific DOM compartments in aquatic systems can be unraveled. What complicates the picture, is that the chemical signature of biorecalcitrant DOM can result from strong microbial reworking. Therefore, combining DOM molecular characterization with bacterial activity measurements would certainly foster the understanding of DOM transformations in highly complex and natural river systems.

In this study, water samples were collected bimonthly in a longitudinal as well as lateral direction from Europe’s last large natural river, the River Tagliamento, Italy (Fig.1). In a comprehensive study, riverine DOM was isolated by C18-solid phase extraction and ultrafiltration to allow for a more complete recovery and characterization of different DOM components, and chemically characterized. Novel information on the spatial-temporal variability in DOM structure is obtained as are downstream changes in DOM molecular composition. We also report here on the bacterial utilization of hydrophobic and hydrophilic, HMW and LMW DOM. Thus, we successfully relate DOM composition to its bioavailability to the natural bacterioplankton community. Overall, this study was performed to (i) elucidate how the chemical features of DOM relate to its origin and bioavailability, and (ii) how this affects the spatial-temporal cycling of riverine C.

Materials and methods

Study area and sample collection
Since 1997 the whole river corridor of the Tagliamento River has been extensively studied (Ward et al. 1999b, Gurnell et al. 2000, Arscott et al. 2000; Karrenberg et al. 2002; van der Nat et al. 2002; Kaiser et al. 2003a; 2003d). The river is classified as the last large natural river in Europe flowing unrestrained from the alpine headwater reaches to the northern Adriatic Sea (Ward et al. 1999b) (Fig. 3.1). Its highly complex morphology and channel structure is characterized by constrained, braided, and meandering reaches formed by a dynamic hydrological regime. The Tagliamento River experiences two major floods within a year (spring and fall), facilitating the deposition and mobilization of materials and leading to major changes in organic matter input and transport.
Figure 3.1
Catchment site of the Tagliamento River with important sampling stations marked by large grey patches: Main channel in headwater floodplain (R2MC), in major floodplain (R4MC), and in transition floodplain (R5MC), and isolated pool in major floodplain (R4P). Find the most important tributary streams, the Arzino, But, Degano, and Fella in the catchment area. The little inset shows that the river is located northeast Italy, Europe.

The water samples for C18-solid phase extraction and ultrafiltration were collected bimonthly from the main channels (MC) in the island/bar-braided headwater floodplain (R2), in the island/bar-braided lower and major floodplain (R4), and in the braided-to-meandering transition floodplain (R5) as well as from one isolated pool (P) located in the major floodplain (R4) of the Tagliamento River (Fig. 3.1). Field work took place during March, May, July, August, October, and November 1999 and April 2000. Water levels during sampling varied from low- to high-flow and flood conditions (Table 1). Over the year, the main channel river water was characterized by a temperatures of
10.9 ± 2.5°C, by pH’s 8.13 ± 0.14, and alkalinities of 2.8 ± 0.5 mmol l⁻¹. Water samples for DOM fractionation were collected with clean 50 l high-density polyethylene (HDPE) carboys and transported back to the field laboratory, located near R4. Immediately following collection, water samples were passed through pre-combusted (250°C) GF/F- and 0.2 µm Durapore filters (142 mm diameter, Millipore) and stored in clean 50 l carboys. The carboys, teflon-lined filtration unit, and silicone and Tygon tubes were thoroughly cleaned with 1 M hydrochloric acid and MQ-UV water (Millipore).

**DOM fractionation and isolation**

DOM was either chemically or size fractionated, except in April 2000, when both methods were employed in parallel. For chemically fractionating DOM into hydrophobic- and hydrophilic-type compounds, a Mega Bond Elute C18 column (C18 loaded silica, 60CC, Varian) was used after acidification with 32% hydrochloric acid (Suprapur) to pH 2.8 (Louchouarn et al. 2001). Before fractionation, the water was acidified. Flow rates range from 3 - 5 L h⁻¹. Of the total DOC (DOC <0.2 µm), 13 - 54% was recovered as a sorbed hydrophobic fraction (Table 1), being comparable to other literature values (Mills and Quinn 1981; Amador et al. 1990). A Filtron tangential flow ultrafiltration system with a polyethersulfone membrane (1 kDa nominal weight cutoff) was used for fractionating bulk DOM into pseudo HMW and LMW portions, following the protocol of Benner et al. (1997). Operating pressures were 137.9 - 151.7 kPa at the inlet and 68.9 - 89.6 kPa at the outlet. Filtration rates range between 6 - 8 l h⁻¹ using one 0.46 m² cassette filter (Centrasette, Filtron). The water temperature ranged from 20 - 22°C during ultrafiltration. Recovery was 8 - 29% of the total DOC (Table 3.1). For every sample, a carbon mass balance was established to determine whether carbon was lost or gained during fractionation. The percentage of DOC retained after fractionation from bulk DOC (<0.2 µm) was calculated as follows: % of initial DOC (<0.2µm) = 100 (DOC_{retentate} + DOC_{permeate})/(DOC_{<0.2µm})², where DOC stands for DOC concentration and was corrected by the according concentration factor (Table 3.1). Mass balance calculations revealed that from initial DOC 99 – 144% was recovered after ultrafiltration and 100 – 139% after C18-solid phase extraction.
From fractionation, samples were stored at 4°C in the dark and immediately transported back to the laboratory in Switzerland (EAWAG). DOM adsorbed onto the C18 phase was eluted by gentle vacuum-filtration using high-purity methanol (Merck). The methanol was removed from DOM by roto-evaporation and freeze-drying. Next, the extract was redissolved in MQ-UV water (Millipore). The aqueous solid phase extracted and ultrafiltered DOM samples were cation-exchanged (Bio-Rad, AG 50W and AG MP-50) to remove trace metals (Everett et al. 1999), and then concentrated by freeze-drying for further chemical and molecular characterization. Tar-like ultrafiltered DOM was redissolved in methanol (except from April 2000) and freeze-dried. However, we could not reach solid phase or completely remove all methanol from the ultrafiltrate, as shown by the intense resonance at 55 ppm in the solid-state NMR spectra (see NMR analyses). Redissolution in MQ-UV water, neutralization to pH 8.1, and subsequent freeze-drying resulted in reaching a solid phase. The dried material was scraped from the Teflon-beakers and stored sealed in pre-combusted glass tubes (Supelco) at 4°C in the dark for chemical analyses.

**TOC and DOC measurements**

Water samples (20 ml) were collected directly after fractionation for ultrafiltered samples and after freeze-drying and redissolution in MQ-UV water for solid phase extracted DOM. The samples were filled in acid-rinsed, pre-combusted 40 ml EPA glass vials, sealed with Teflon-lined caps, and stored frozen until analysis. Total organic carbon (TOC) and DOC concentrations were determined by high temperature catalytic oxidation with a Shimadzu 5050A analyzer (Benner et al. 1993; 1997).

**Size exclusion chromatography (SEC)**

Water samples for SEC were collected in acid-rinsed, pre-combusted 40 ml EPA glass vials (Wheaton), sealed with Teflon-lined caps, and stored frozen until analysis. Gel (TSKHW50S) permeation chromatography with UV-absorbance (at 254 nm) and DOC detection was used to characterize the apparent molecular weight distribution of the DOM fractions (Mueller et al. 2000). The mass-selective detection of DOC is based on the absorption of infrared light by CO₂ from photooxidized DOC. The mobile phase was
composed of “carbon-free” water (UV-photooxidized MQ-UV water, Millipore), 3.6 mM sodiumhydrogenphosphate, and 18.4 mM potassiumhydrogenphosphate (pH 6.6). Potassiumhydrogenphosphate was also used for calibration. A variety of organic model substances were employed to describe the size exclusion of different molecular weight ranges between 200 – 0.1 kDalton (kDa) (Huber and Frimmel 1992). Humic substances, LMW organic acids, polysaccharides, proteins, and amphiphilic and hydrophobic organic substances were used for qualitative and quantitative interpretations of chromatograms of natural DOC. We calculated the molecular size distribution by methods described by Perminkova et al. (1998).

**One-dimensional (1-D) solid-state ramp $^{13}$C CPMAS-NMR**

NMR spectra were obtained as described by Zang et al. (2000) and Dria et al. (2002) using the ramp cross polarization magic angle spinning (ramp CPMAS) pulse program and two pulse phase modulated (TPPM) decoupling on a Bruker DSX 300 NMR spectrometer, operating at a frequency of 300 MHz for $^1$H or 75.48 MHz for $^{13}$C. Approximately 20 - 30 mg of sample was placed in a 4 mm (outside diameter) NMR rotor with a Kel-F® cap. Samples were spun at a frequency of 13 kHz using a contact time of 2 ms and a 1 s recycle delay time. For our samples 80,000 - 100,000 acquisitions (scans) were collected. For each sample’s free induction decay, 1,024 complex data points were collected and zero-filled to a total of 4,096 data points. The samples were Fourier-transformed, 100 Hz line-broadening was applied and phased appropriately. The carboxyl carbon of glycine (176.03 ppm) provided a secondary reference for all solid-state spectra.

The spectra were split into aliphatic and aromatic regions and integrated over 0 - 60, 60 - 90, 90 - 110, 110 - 160, 160 - 180, and 180 - 230 ppm regions for quantitative comparisons. Areas of peaks not associated with those from ultrafiltered DOM (at 55 ppm from methyl esters and 0 ppm from siloxanes) were subtracted from the integrated area. The aliphatic region includes the integrals between 0 - 110 ppm, and the area between 110 - 160 ppm was assigned to aromatic carbons (Malcolm 1990). The aromaticity and aliphaticity of each sample was calculated as a percent of the total integrated area (Table 3.1).
**TMAH thermochemolysis GC/MS analyses**

Lyophilized DOM originating from C18-solid phase extraction and ultrafiltration was used for thermochemolysis following the protocol of Hatcher and Clifford (1994), del Rio et al. (1998), and Frazier et al. (2002). Approximately 1 mg of organic matter was weighed out into a glass ampoule with 200 µl of TMAH (25% in methanol). The methanol was evaporated under a stream of nitrogen. The ampoules were sealed under vacuum and subsequently baked at 250°C for 30 minutes. After cooling, the ampoules were scored, frozen in liquid nitrogen, and then broken open. At the same moment 35 µl of an internal standard solution (23.0 ppm eicosane in ethyl acetate) was added to each ampoule (805 ng eicosane). The products were extracted from the ampoule with 1.3 ml of ethyl acetate and concentrated under nitrogen to approximately 100 µl in a GC vial prior to analysis.

Qualitative analyses were performed with a Hewlett-Packard 6890 GC with a split-split-less injector operating in the split mode and utilizing helium as the carrier phase. The GC was fitted with a 15 M Rtx-5MS capillary column (250 µm I.D., 0.1 µm film thickness, Restek). This system was coupled to a Pegasus II (Leco Corporation) time-of-flight mass spectrometer operated in E.I. mode with a filament bias of –50 eV. The source was maintained at 200ºC and the transfer line at 280ºC. Peak identifications were based on comparisons with standards and the NIST library (version 1.6). The GC was operated with a constant inlet temperature of 280ºC and the oven programmed to heat from 40ºC to 210ºC at a rate of 8ºC min⁻¹ and then from 210ºC to 300ºC at a rate of 25ºC min⁻¹, holding for one minute at the minimum and maximum temperatures. Mass spectra were collected at a rate of 17 spectra per second after the 110-second solvent delay. Masses were acquired from m/z 35 to 450.

**Bacterial abundance and production**

Samples (10 ml) for microscopy were preserved with a filtered borax-buffered formaldehyde solution (5% final concentration) and stored refrigerated in the dark. Bacterial abundance was determined within two weeks of sample collection by epifluorescence microscopy (Olympus BX50, 1,000 magnification) of 4’,6-diamidino-2-phenylindole (DAPI)-stained cells (Porter and Feig 1980). In the lab and field
experiments, bacterial production was estimated from protein synthesis as measured in triplicate with two formalin-killed blanks (2% final concentration) by adding $[^3H]$leucine (20 nmol l$^{-1}$ final concentration) to each of the 5 ml subsamples and incubating them in the dark at 20ºC for 60 minutes (Kirchman 1986). After incubation, the samples were filtered using cellulosenitrate filters (GSWP, 0.2 µm pore-size, 25 mm diameter, Millipore) and rinsed twice with 5 ml of chilled 5% trichloroactetic acid. The filters were placed in scintillation vials with 10 ml of scintillation cocktail (Insta-Gel, Packard) added. Radioactivity was assessed with a liquid scintillation counter (Packard Tri-Carb 2000) by external standard ratio technique. We used the factor 3.1 µg C nmol$^{-1}$ leucine for converting leucine incorporation into the bacterial biomass (Simon and Azam 1989).

**Results**

**Abundance and chemical composition of DOC**

Over the year, in the Tagliamento River, the dominant form of organic carbon was always in dissolved form. Downstream DOC concentrations were highest in R4MC and lowest in R5MC and R4P (Table 3.1, Fig. 3.1). Further, we observed seasonal fluctuations in DOC concentrations as well as sharp changes due to flood/non-flood conditions. Over the year, concentrations of bulk DOC (<0.2 µm) ranged from 38 - 89 µmol C l$^{-1}$ in R2, from 39 - 95 µmol C l$^{-1}$ in R4, and from 32 - 83 µmol C l$^{-1}$ in R5 mainstem surface waters, and from 35 - 66 µmol C l$^{-1}$ in R4 pool surface waters, with an overall average (± standard error, SE) value of 69 ± 5 µmol C l$^{-1}$ (Table 1).

Along the river continuum, R4 and R5 exhibited largest fluctuations in DOC concentrations during spring and winter (Table 3.1). DOC concentrations were highest in March 1999 and April 2000, corresponding to high-flow conditions. Conversely, in July 1999 the water flow ceased and the DOC values strongly dropped. Hence, during summer the river system became very homogeneous, exhibiting almost equally low amounts of DOC present in all reaches. We observed a relatively small increase in DOC levels after a flood event in August and during high-flow regimes in November 1999. Nevertheless, this flood event disrupted summer low-flow conditions and low DOC concentrations, and
supplied a short-term moderate spike of C to the system. The DOC concentrations again slightly increased within the second half of the year 1999.

The percentage of surface water DOM retained by C18-solid phase extraction and by ultrafiltration annually ranged from 13 - 54% and 8 - 29% of the bulk DOM (<0.2 µm), respectively (Table 1). The annual average (± SE) percentages retained by C18-solid phase extraction were 26 ± 8% for R2MC, 27 ± 4% for R4MC, 34 ± 8% for R4P, and 34 ± 11% for R5. The annual average (± SE) percentages retained by ultrafiltration were 19 ± 9% for R2MC, 22 ± 11% for R4MC, 18 ± 10% for R4P, and 18 ± 6% for R5MC.

**Molecular size distribution of DOM**

HMW, LMW, hydrophobic, and hydrophilic DOM, processed from one large water sample collected in R4MC in April 2000, were analyzed by SEC for their apparent molecular distribution (Fig. 3.2). Chromatograms are difficult to interpret because SEC separates organic substances according to molecular size, but also to hydrophobic and ionic interactions (Chin et al. 1994). Therefore, substances with high molecular size may possibly represent DOM aggregation (unpublished data). We calculated that for these samples over 97% of the initial DOC was recovered after passing through the gel chromatography column. The HMW DOM consisted of organic compounds ranging from 80 - 1 kDa, likely representing different carbohydrates, proteinaceous and aliphatic/fatty acid material, and some LMW organic compounds (Kaiser et al. 2003a). The major components sized 20 - 2 kDa and peak values were found at 50, 5, and 2 kDa. It was interesting that UV detection at 254 nm (Fig. 3.2 B) showed no absorbance for HMW DOM 50 kDa in size, which would indicate the presence of polysaccharides or glycosilated proteins. LMW DOM ranged from 10 - 0.5 kDa, with peak values at 5 and 0.7 kDa, probably indicative of LMW organic acids, oligosaccharides, and peptide material (Fig. 3.2 A). It is interesting to note that structures up to 7 kDa can be found in the <1 kDa ultrafiltration permeate. These substances are larger compounds that managed to pass the 1 nm-sized pores of the ultrafiltration membrane, probably due to their linear structure. The UV absorbance for LMW compounds 1 - 0.5 kDa in size were strongly reduced, again indicative of sugars (Fig. 3.2 B).
Hydrophobic DOM eluted in a similar fashion to HMW DOM (Fig. 3.2 A), however, showed a narrower size spectrum ranging from 20 - 0.5 kDa and no peak value at 50 kDa, but increased UV absorbance from 1 - 0.5 kDa (Fig. 3.2 B). Hydrophilic DOM eluted different than LMW DOM (Fig. 3.2 A), exhibiting two peaks at 10 - 0.5 kDa and < 0.1 kDa, thus including larger and very small units, which possibly (by comparison with the standards) contain peptides, sugars, LMW organic acids, and amino acids. Only the larger compounds exhibited UV absorbance (Fig. 3.2 B), indicative for the presence of sugars. Interestingly, approximately 50 kDa size DOM (such as found in HMW DOM) was not found in either hydrophobic nor hydrophilic DOM. We explain the absence of these larger molecules in this fraction by hydrolysis due to acidification required for C18-solid phase extraction. Glycosilated proteins and polysaccharides associated with that molecular weight range would easily hydrolyze at low pH, as discussed above.

Fig. 3.2
Size exclusion chromatograms for different DOM fractions from April 2000. The apparent molecular weight distribution of DOM was determined by (A) DOC and (B) UV (254 nm) detection.
Bulk chemical characterization with solid-state $^{13}$C NMR

The $^{13}$C NMR spectra of solid phase extracted hydrophobic and ultrafiltered HMW DOM isolates showed broad resonances and had relatively low signal-to-noise ratios due to low sample quantities, sample complexity, and low organic C contents. However, the spectral qualities are quite reasonable when compared to other published DOM solid-state $^{13}$C NMR spectra (McKnight et al. 1997). Spectra obtained by both extraction techniques exhibit four major functional groups of organic compounds: aliphatics, carbons adjacent to O or N (such as in carbohydrates, amino acids, esters, alcohols, etc.), aromatics, and carboxyls-/aliphatic amides. The chemical shifts of more defined signals indicated specific functional groups or structures, such as: Alkyl groups (12 - 25 ppm), methylene (29 - 33 ppm), sugars, aliphatic methine, and methoxy-like $\alpha$–amino acids and alcohols (45 - 90 ppm), anomeric carbons of polysaccharides (90 - 110 ppm), aromatics and alkenes (110 - 140 ppm), aromatic heterocycles (140 - 160 ppm), carboxylates, aliphatic amides (160 - 190 ppm), carbonyls and ketones (190 - 230 ppm).

These spectra exhibit some sharp peaks that are uncharacteristic of DOM and may be attributed to experimental artifacts. The sharp clipped peaks observed in the hydrophobic DOM spectra (~33 ppm in Figure 3.3, 3.4, 3.5) can be attributed to C18 contamination during the solid phase extraction process. HMW DOM spectra (Fig. 3.5) show intense peaks at 56 ppm, which result from methyl ester artifacts formed during the process of trying to obtain a solid material by methanol extraction under acidic conditions. Spectrum H in Fig. 3.5 contains a small 56 ppm peak because this sample was not treated with methanol. The 0 ppm peak observed in the ultrafiltered DOM spectra (Fig. 3.5) is likely attributed to methyl siloxanes of undetermined origin.

Ignoring the contaminant peaks, little variability is observed on a spatial-temporal gradient (Fig. 3.3, 3.4, 3.5), while differences are observed between the two extraction techniques (Kaiser et al. 2003a). All spectra are displayed with the same carboxylic acid/aliphatic amide (160-190 ppm) signal intensities. The hydrophobic DOM samples contain very high aliphatic C contents, high carbohydrate/alcohol contents and relatively low aromatic C concentrations. Ultrafiltered DOM spectra have considerably greater aromatic C contents, lower aliphatic C and lower carbohydrate/alcohol C contents, and the presence of some methoxy/amino group carbons. These aromatics show phenolic
signals indicating the contribution of lignin to these samples. Table 3.1 lists all the aliphatic:aromatic ratios calculated for the individual DOM isolates. Solid phase extracted DOM in all cases exhibited higher aliphatic:aromatic ratios than ultrafiltered DOM.

Variability in the chemical composition of solid phase extracted DOM was low throughout the river (Fig. 3.3, 3.4; only March, July, October, and April are shown). In March, R4MC (Fig. 3.3 B) showed an elevated amount of aromatic and methoxy/amino group carbons compared to the other reaches, likely attributed to lignin. In July, R5MC (Fig. 3.3 H) revealed an increased carbohydrate/alcohol content. In general, slightly higher aliphatic:aromatic ratios were calculated for R2MC and R4P, compared to R4MC and R5MC (Table 3.1). Interestingly, seasonal variability in DOM composition was also low. When comparing hydrophobic DOM from R4MC (collected in March, July, and October 1999 and April 2000) we could observe a decrease in the relative signal intensities for the aromatic and especially the methoxy/amino group carbons from March to July 1999, and a prevalence of the carbohydrate/alcohol content in the same months (Fig. 3.5). In October 1999, the relative proportions of aromatics increased and of carbohydrates decreased relative to July, but both were still less abundant compared to March. The other aliphatic compounds increased relative to March and July. The aliphatic:aromatic ratios were lowest in March, then gradually increased throughout the seasons, and reached again slightly lower numbers in October (Table 3.1), but overall variations were small. Spectra from ultrafiltered HMW DOM from R4MC (collected in May, August, and November 1999 and April 2000) showed very similar trends (Fig. 3.5). In April and November, aliphatic compounds were more dominant, with higher aliphatic:aromatic ratios compared to other months (Table 3.1). April also showed the largest carbohydrate peak of all the HMW samples. During a flood in August, the relative signal intensities of all C functional groups decreased relative to the carboxyl peak (175 ppm). In November, we detected a higher content of aromatics and thus lower aliphatic:aromatic ratios.
Fig. 3.3
Solid-state ramp $^{13}$C NMR spectra measured from hydrophobic solid phase extracted DOM sampled in a longitudinal direction from the main channel in March and July 1999: (A and E) Headwater floodplain (R2MC), (B and F) major floodplain (R4MC), (C and G) isolated pool in major floodplain (R4P), and (D and H) transition floodplain (R5MC). Contaminant peak (*) at ~33 ppm is attributed to C18 contamination during solid phase extraction.
Fig. 3.4
Solid-state ramp $^{13}$C NMR spectra measured from hydrophobic solid phase extracted DOM sampled in a longitudinal direction from the main channel in October 1999 and April 2000: (A and E) Headwater floodplain (R2MC), (B and F) major floodplain (R4MC), (C and G) isolated pool in major floodplain (R4P), and (D and H) transition floodplain (R5MC). Contaminant peak (*) at ~33 ppm is attributed to C18 contamination during solid phase extraction.
Fig. 3.5
Seasonal solid-state ramp $^{13}$C NMR spectra measured from (A) hydrophobic and (B) HMW DOM, which were isolated from the main channel in the major floodplain (R4MC) or in the transition floodplain (R5MC). Contaminant peak (*) at ~33 ppm is attributed to C18 contamination during solid phase extraction, and (**) at 0 ppm is attributed to methyl siloxanes.
Molecular characterization with TMAH thermochemolysis

To determine downstream differences in DOM molecular composition, we utilized solid phased extracted and HMW DOM isolates sampled from R4MC and R5MC in April 2000. For both locations, we have isolated the two different DOM fractions from one water sample. In solid phased extracted DOM from R4MC and R5MC we have identified different aromatic, higher plant biopolymer degradation products, and a series of fatty acid methyl esters (FAMEs) (Table 3.2; Fig. 3.6 A, C).

In R4MC, early eluting compounds are polysaccharide-derived compounds, such as methyl- and methoxy-cyclopenten-1-ones, methoxy-pentenone, and ethanone as well as ‘general’ DOM products, such as butanedioic and pentanedioic acid. In R5MC, we could only detect cyclopentenone and similar ‘general’ DOM products. Aromatic TMAH products and N-containing compounds mainly eluted in the mid-part of the chromatograms. In R4MC DOM, the aromatic syringyl derivatives are trimethoxytoluene and trimethoxybenzoic acid methyl ester (S2, S6), and the guaiacyl derivative is dimethoxybenzene (G1). R5MC additionally displayed the lignin-derived product trimethoxybenzene (S1), the guaiacyl derivative dimethoxybenzaldehyde (G4), and the p-hydroxyphenyl-derived compound methoxy benzoic acid methyl ester (P6). But the aromatic compounds dimethoxybenzene, trimethoxybenzene, and trimethoxybenzoic acid methyl ester may also be produced from the degradation of other biomolecules, such as polysaccharides, tannins or proteins (Fabbri and Helleur 1999, Frazier et al. 2001). In R5MC, we further observed trimethoxybenzene, which may originate from cutan, an epicuticular hydrophobic plant polymer. For both DOM isolates the observed N-containing compounds may be protein-derived. However, some heterocyclic molecules, possibly pyrrolidinediones (N1, 2), could also represent artifacts from the TMAH reaction. A series of FAMEs were present throughout the chromatograms, ranging from octanoic acid to octadecanoic acid methyl ester. These even-numbered saturated, monounsaturated, and branched iso- and anteiso-fatty acids are typically lipid-derived and indicate different biological sources, ranging from prokaryotic to eukaryotic (algal) origin. Uneven-numbered fatty acids originate from the thermochemolysis process. Branched iso- and anteiso-fatty acids were only present in R5MC and indicate a bacterial origin. We also noted the presence of chloro-organic substances, which may originate
from pulp-mill effluents. We recovered chlorobenzoic acid methyl ester and chloro-methoxy benzoic acid methyl ester in R4MC, and chloro-dodecane and chloro-tetradecane in R5MC.

HMW DOM from R4MC showed much better recovery of organic compounds than from R5MC. We have identified variable aromatic lignin-derived compounds, higher plant biopolymer degradation products, and a series of fatty acid methyl esters (FAME) in ultrafiltered DOM from R4MC and to a small extent from R5MC (Table 3.3; Fig. 3.6 B, D). In DOM from R4MC, early eluting compounds are polysaccharide derived compounds, such as methyl-cyclopentene-1-one, cycloheptene, dimethyl-cyclopentene-1-one, and furan, and ‘general’ DOM products, such as butanedioic and pentanedioic acid, the syringyl derivative trimethoxybenzene (S1), and octanoic acid methyl ester. In R5MC we only detected trimethoxybenzene, butanedioic acid, benzoic acid methyl ester, and pentanedioic acid. For R4MC DOM, mainly N-containing compounds and lignin-derived products were observed in the chromatograms (Table 3.3). For both DOM isolates, we also believe that the N-containing compounds may stem from protein, based on the presence of protein/peptide material identified by multidimensional solution-state NMR in the DOM from R5MC (Kaiser et al. 2003a). The aromatic syringyl derivative is trimethoxybenzene, (S1), and the guaiacyl derivative is dimethoxybenzene (G1). A series of FAMEs were present throughout the chromatogram, ranging from heptanoic acid to octadecanoic acid methyl ester.
Total ion current traces from thermochemozytes from hydrophobic-type and ultrafiltered DOM isolated (A, C) from the main channel in the major floodplain (R4MC), and (B, D) from the transition floodplain (R5MC) in April 2000.

**Bacterial utilization of different DOM fractions**

In our uptake experiments, bacterial production rates from radiolabeled leucine incorporation were consistently higher on LMW and hydrophylic DOM fractions as compared to HMW and hydrophobic DOM fractions (Fig. 3.7). Over the year, the average peak value of $254 \pm 72$ fg C cell$^{-1}$ d$^{-1}$ was measured from LMW DOM sampled from R4MC, and the average minimum value of $14$ fg C cell$^{-1}$ d$^{-1}$ from hydrophobic DOM sampled from R2MC as well as R5MC. However, for each reach, mean production rates were calculated from bimonthly measurements, because the variability in bacterial production on the individual DOM fractions was minimal over the year.
Fig. 3.7
Average bacterial biomass production on different organic matter fractions bimonthly sampled from (A) R2MC, (B) R4MC, (C) R4P, and (D) R5MC in the Tagliamento River. Mean turnover times (days) for different organic matter fractions were determined according to the following formula: Turnover time = TOC (or DOC)/(bacterial production × 5). Data represent averages of 7 experiments; vertical bars indicate standard errors. Horizontal columns represent bacterial biomass production, and points (●●) represent turnover times.

Bacterial utilization of the individual DOM fractions partially led to changes in the molecular size distributions (Fig. 3.8). We could detect little removal of hydrophobic DOM within 75 hours of bacterial growth (Fig. 3.8 A), as reflected by a decrease in signal intensities. The result was similar for hydrophilic DOM. Bacteria did not efficiently utilize this DOM fraction within a period of 25 hours, and microbial degradation only led to the production or release of very small molecules with molecular weights <0.1 kDa (Fig. 3.8 B). For HMW DOM, SEC revealed a preferential removal of
compounds 70 - 30 kDa in size and a relatively small augmentation in compounds with molecular weights 10 - 4 kDa and 1 - 0.7 kDa within 75 hours of bacterial growth (Fig. 3.8 A). From LMW DOM, bacteria preferentially consumed 2 - 1 kDa size compounds sizing and released compounds <0.1 kDa within 50 hours of growth (Fig. 3.8 B).

Fig. 3.8
Size exclusion chromatograms of four different DOM fractions measured by DOC detection. (A) Solid phase extracted DOM, (B) hydrophilic DOM, (C) HMW DOM, (D) and LMW DOM. Solid lines represent bacterial growth (A) on solid phase extracted DOM after 75 hours, (B) on hydrophilic permeate after 50 hours, (C) on HMW DOM after 75 hours, and (D) on LMW DOM after 50 hours of bacterial inoculum. Samples were taken after the bacterial growth had reached steady state.
Discussion

Abundance and distribution of DOC

Surface water DOC concentrations measured from the Tagliamento River were overall very low (38 - 95 µmol C l⁻¹) (Table 1) and comparable to other highly oligotrophic aquatic systems (Benner et al. 1997; Tockner et al. 2002). We explain the highest concentrations in the major floodplain (R4) by large inputs of plant and soil organic biomass, resulting from a tight coupling of aquatic and terrestrial sites compared to other reaches (van der Nat et al. 2002). In contrast, dilute DOC concentrations at site R4P likely result from massive groundwater upwelling, continuously supplied by an almost 100 m deep alluvial zone to the surface system. The alluvial zone tightly links all water bodies and certainly impacts their DOM signature (Ward et al. 1999b).

DOC concentrations in fluvial systems usually fluctuate with discharge (Mantoura and Woodward 1983) (Table 3.1). Interestingly, in the Tagliamento River the DOM concentrations showed only nominal increases during flooding in August and high-flow regimes in November (Table 3.1). During the flood event discharge was high, and hence, also export of particulate solids. However, by using x-ray diffraction we found that the increased particle load was predominantly inorganic in its composition (calcite and dolomite) and was strongly depleted of organic carbon (unpublished data). Therefore, during floods only negligible amounts of DOC are adsorbed to particles and deposited or exported.

Compared to other lotic systems, the Tagliamento River carries low amounts of hydrophobic or HMW DOM (Kaplan et al. 1980; Thurman 1986; Hedges et al. 1994). On average, 30% could be isolated by C18-solid phase extraction and 17% by ultrafiltration from the surface water samples (Table 1). Even from groundwater only 32% of HMW compounds could be retained (data not shown). These recoveries showed surprisingly minimal variability in a downstream direction and during seasonal sampling. We attribute this uniform spatial-temporal chemical characteristics to fast longitudinal processing of bioavailable LMW and hydrophilic DOM and transport of biorecalcitrant and degraded HMW and hydrophobic DOM (see below).
Bulk chemical composition of DOM isolates

SEC and $^{13}$C NMR show that the four representative DOM fractions covered different molecular weight ranges (Fig. 3.2 A; Fig. 3.5 D, H), and suggest that solid phase extraction and ultrafiltration select for different suites of molecules (Kaiser et al. 2003a). Although forming a small portion of bulk DOM, HMW DOM covers the largest spectrum of molecular weights, with carboxyls, aromatics, low levels of aliphatic/fatty acid material, relatively low levels of carbohydrate/O alkyl carbons, and some methoxy/amino group carbons (Fig. 3.5). We additionally have evidence for abundant peptide/protein material present (Kaiser et al. 2003a). Conversely, LMW DOM being the dominant portion of DOM separated by ultrafiltration, covered a narrow window of molecular weights (Fig. 3.2 A). We still have little knowledge about its chemical composition, but it appears that it mostly contains LMW organic acids, oligosaccharides, amino sugars, and amino acids. We further measured a low average C:N ratio of 7 for bulk DOM and 34 for HMW DOM. By difference we can calculate an average C:N ratio of 5 for LMW DOM, consistent with the presence of proteinaceous material (unpublished data).

Hydrophobic DOM demonstrated a smaller apparent molecular weight distribution than HMW DOM (Fig. 3.2 A), with high aliphatic C contents, high carbohydrate/alcohol contents, and relatively low concentrations of aromatics (Fig. 3.3, 3.4, 3.5). Hydrophilic DOM contains proportions of compounds with low molecular weight, which we assume are comprised of sugars, fragments of hydrolyzed glycosilated proteins (see results), and LMW organic acids. The presence of peptide/protein material is suggested from the relatively high UV absorbance of the large molecular weights, and from low average C:N ratios (3.3) of this fraction (data not shown).

$^{13}$C NMR of hydrophobic and HMW DOM reveal minimal downstream changes in DOM composition during October 1999 and April 2000 (Fig. 3.4, HMW DOM not shown), with noticeable variations only detected in March and July 1999 (Fig. 3). Winter low-flow and low temperature conditions cause an accumulation of organic matter in the vast alpine catchment (Fig. 3.1). Freezing also diminishes leaching of soil biomass from surrounding soils and sediments. Therefore, in winter, less DOM is released to Tagliamento surface waters. In spring, rising temperatures and melt-water from the Alps (Arscott et al. 2000) again induce transport and release of aged and diagenetically altered
DOM from terrestrial biomass and soils to surface river water. The abundance of aromatic and methoxy carbons (indicative for lignin) in R4MC in March points to influx of decomposed lignin-rich plant biomass (Fig. 3.3).

In contrast, in July, site R5MC experiences a relatively high contribution of sugar functionalities to mainstem waters, which implies either strong microbial reworking of cellulose and hemi-celluloses, stemming from terrestrial biomass, or autochthonous biomass production from primary productivity along the river. Some studies from the literature suggest a prevalence of algal produced carbohydrates during spring and summer (Kaplan et al. 1980). However, in the Tagliamento River, in these seasons chlorophyll$_a$ concentrations were at the limit of detection in surface waters (Arscott et al. 2000) and planktonic microalgae production was phosphorous (P)-limited (Arscott et al. 2000; Kaiser et al. 2003b). Additionally, we were not able to infer any dominance of sugars in April 2000. Thus, in July 1999, the observed surplus of sugars may dominantly derive from benthic algal production. In April 2000, benthic algal production was still suppressed or downstream processing of released carbohydrates was fast. Interestingly, flooding in August had no significant impact on DOM composition (data not shown), and reconfirmed that high discharge may also contribute to low downstream variability in DOM composition.

**Molecular composition and origin of DOM isolates**

DOM structures and bacterial DOM utilization (discussed later) suggest that longitudinal processing of bioavailable DOM is fast and more effective than seasonal transformations. For this reason, we resolved possible molecular changes in DOM composition between two downstream reaches (R4, R5).

Polysaccharide-derived compounds in DOM isolates, indicative of vascular plant origin (Frazier et al. 2003), were removed downstream. The fact that surface water flow is fast between R4 and R5 (Arscott et al. 2000) strengthens the assumption that these sugars are readily available to the microbial community. Downstream loss of sugars correlates well with the preferential utilization of sugars from HMW DOM (see also bioassays, Fig. 3.8 C). The N-containing compounds recovered likely originate from peptide/protein material (Kaiser et al. 2003a). N-containing compounds show no
longitudinal variability reflecting their recalcitrant chemical nature, possibly resulting from strong diagenetic alteration. Low bacterial growth on DOM isolates (discussed later, Fig. 3.8 A, D) also support that these compounds are persistent against microbial attack. Kaiser et al. (2003a) hypothesized that refractory ligno-protein complexes, encapsulated protein structures, or sugar-crosslinked amines may form this recalcitrant portion of proteinaceous riverine DOM.

The diversity of lignin-derived products increased downstream (Table 3.2). Its composition suggests predominant woody angiosperm origin. In R4MC and R5MC, the riparian vegetation is mostly composed of angiosperm biomass, particularly Alnus, Populus, and Salix (Karrenberg et al. 2002b). Lateral erosion donates the largest portion of plant organic matter, such as wood and leaf biomass, to the river system (Van der Nat et al. 2002). However, approximately 30% of organic matter originates from higher reaches and the vast alpine catchment area, also containing dominantly angiosperm vegetation (Van der Nat et al. 2002; Karrenberg et al. 2002b) (Fig. 3.1). Therefore, part of the lignin-material present in the lower floodplains is transported over large distances within the Tagliamento River. Due to floodplain complexity, the river has a high capacity to retain terrestrial biomass (Gurnell et al. 2000), and decomposition processes occur not only during fluvial transport, but also during storage. Interestingly, it was found that wood decomposition happens slightly faster in aquatic sites than on land (Van der Nat et al. 2002). But still, although organic matter load is high in the river system, the abundance of aromatic methoxy compounds was low in surface waters. The dominance of acids (Table 3.2, 3.3) additionally points to a presence of microbially degraded DOM, because these acids are produced due to microbial oxidation of α-carbons of lignin-derived aldehydes (Hedges et al. 1997). Hence, we assume that the lignin decomposition products partially stem from the headwater regime, with downstream transport involving microbial reworking, and partially from the floodplains, where long-term storage and decomposition lead to high diagenetic alteration.

The FAME signature of DOM isolates shows that bacterial biomass (gram-negative bacteria) mainly contributes to riverine DOM. The FAMEs composition undergoes small downstream variation, which relates to a non-reactive character of the lipids present or a steady production of the same distribution. Potential leaching of “old”
and biorecalcitrant lipids from soil biomass to Tagliamento surface waters (Kaiser et al. 2003a) and low bacterial growth on DOM isolates help to explain this non-reactive nature (see later). There is little contribution from eukaryotes as expressed by the low contribution of polyunsaturated fatty acids. In the Tagliamento River, microalgae biomass is low due to high P-limitation (Arscott et al. 2000) and its degradation to small organic compounds is rapid, as suggested by the high abundance of hydrophilic and LMW DOM.

We also noted the presence of chlorinated organic substances in TMAH products, which are not derived from natural sources, but may originate from anthropogenic waste. We suspect influx of pulp-mill effluents upstream from R4 (Venzone) (Fig. 3.1). Although this seminatural river owes a highly dynamic hydrology (Ward et al. 1999b) and efficiently recycles nutrients (Arscott et al. 2000), these anthropogenic products are transported over long distances. We explain that finding by the refractory chemical character inherent of chlorinated compounds and their little susceptibility to biological degradation under oxic conditions (Holliger et al. 1997).

**Chemical composition and bioreactivity of DOM fractions**

In Tagliamento surface waters the low abundance and growth of small, planktonic cocci indicates severe limitation by annually low temperatures (10.9 ± 2.5°C) and dissolved inorganic P concentrations (Arscott et al. 2000). Considering the low DOC concentrations and the high diagenetic alteration of HMW and hydrophobic-type DOM components, we can further expect an additional C limitation.

On a spatial scale, we have tested the bacterial utilization of different DOM fractions, and found that downstream bacterial activity partially follows the classic ‘river continuum concept’ (Vannote et al. 1980). In the headwaters, except for hydrophobic DOM all other organic matter fractions (including raw water and DOM<0.2 µm) exhibited similar high bioavailability (Fig. 3.7). With longitudinal transport the importance of LMW DOM for bacterial growth increased. Bacterial uptake of LMW DOM was highest at site R4MC, possibly induced by a higher proportion of readily available organic compounds and a higher abundance of inorganic nutrients (Arscott et al. 2000). This finding partially contradicts studies that suggest a downstream decrease in
bioavailability of DOM (Hedges et al. 1986b; Richey et al. 1990). In the isolated pool at site R4, though bacterial DOM utilization was lower compared to that of the mainstem, its seasonal pattern was similar, suggesting similar DOM sources. We explained this effect by high connectivity between different lateral water bodies through intense groundwater upwelling from the deep alluvial zone. The pattern in bioavailability of all organic matter fractions stayed very constant throughout the year 1999 and partially 2000, reflecting invariable temporal DOM composition.

As suggested earlier, organic substrates with different nutritional quality are released into mainstem waters along the river and immediately reworked with downstream transport. Their microbial decomposition dominantly releases LMW and hydrophilic-type DOM and also inorganic nutrients (nitrate, phosphate) (see above), which both may support microbial activity along the river continuum (Fig. 3.7). Based on TOC and DOC concentrations, on bacterial biomass production, and assuming an average growth yield of 20% (Cole et al. 1988; Coffin et al. 1993), we were able to estimate TOC and DOC turnover times (days) in the River Tagliamento (Fig. 3.7). Over the year, the mean turnover time is highest for hydrophobic DOM by ranging from 110 to 204 days, whereas LMW and hydrophilic DOM show turnover times of only 6 to 11 days. Slightly elevated bacterial production on hydrophobic DOM in R4MC paralleling high turnover times may be explained by an overall higher abundance of either bioavailable constituents or dissolved inorganic phosphorous in the major floodplain. Interestingly, the surface water needs approximately 2 to 3 days (estimated from annual mean surface flow) to travel from the spring region to the sea. This implies that more than half of the organic matter transported gets transformed before it enters the sea.

During growth on LMW DOM, bacteria were only utilizing the largest components and transformed them to very small molecules (Fig. 3.8 C). The molecules released while growth on hydrophilic substrates revealed similar small molecular weight (Fig. 3.8 B). Based on SEC and an additional study on organic matter transformations in the Tagliamento River (unpublished data), it appears that in this river microbial decomposition of POM and DOM sources partially follows the so-called size-reactivity-continuum model proposed by Amon and Benner (1996). As proposed fine suspended organic particles are degraded to LMW and hydrophilic DOM and further on to very
small compounds (<0.1 kDa). Diagenesis occurring along downstream transport involves a decrease in molecular weight and possibly also reactivity. We do not know if the very small compounds released are less bioreactive, however, we know that the smallest compounds contained in the LMW and hydrophilic DOM are not supporting bacterial growth (Fig. 3.8 B, D). The fact that the very small products did not absorb UV light (SEC data not shown) suggests the presence of highly degraded organic moieties.

In comparison, hydrophobic and HMW DOM bioavailability was dominantly low throughout lotic transport, exactly reflecting its different origin, invariable chemical composition, and high diagenetic alteration (see molecular characterization). A few exceptions, such as slightly elevated bioavailability of hydrophobic DOM in R4MC samples, may be explained by a higher abundance of either bioavailable constituents or higher concentration of inorganic phosphorus in the major floodplain (see above). HMW and hydrophobic fractions certainly contain a small proportion of bioreactive compounds (Fig. 3.8 A), and therefore, they can only support low bacterial growth. Because their turnover is overall low (see above), these DOM components may have fates other than microbial decomposition while transport in surface waters. They may undergo, e.g., photochemical transformations (Kaiser et al. 2003d).

**Fates of riverine DOM**

In the River Tagliamento, a small portion of DOM compounds, dominantly derived from plant matter and soil biomass, shows low bioreactivity and is mostly transported with surface flow. What drives the river energy and nutrient flow is a diagenetically young and fast cycling pool of LMW and hydrophilic substances. They seem to have different origin and low ages compared to HMW and hydrophobic DOM, which explains their reactive and bioavailable nature. Fast downstream processing of bioavailable compounds adds incrementally to a uniform signature of bulk DOM, along the river continuum and over the year.

From this research we conclude, that organic matter transformations in natural and highly dynamic and heterogeneous river systems are much more efficient than in regulated rivers. Influx of organic substrates is high across the interface of aquatic and terrestrial sites, however, their degradation is also fast. Because ecosystem complexity
helps to retain and rework organic materials (Gurnell et al. 2000), a pool of highly altered and small compounds also enters the river and becomes dominantly transported to its mouth in the coastal ocean. It had been shown that changes in salinity induce precipitation (flocculation) of DOM in the river plume (Sholkovitz 1976). This is only reported for the larger DOM constituents. We do not know what happens to LMW and hydrophilic substances when they enter the marine environment. It had been shown that terrigenous organic matter contained in marine HMW DOM only resides for up to a maximum of 130 year in the ocean (Opsahl and Benner 1997). But still, we do not know the residence time of LMW terrigenous DOM in marine waters, because a high abundance of salts prevents isolation and further chemical characterization. Based on our results, we assume that highly degraded and small freshwater compounds contribute to the pool of highly biorecalcitrant and old marine DOM (Williams and Drueffel 1987).

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References


Table 3.1
Sample descriptions, dissolved organic carbon (DOC) concentrations, mass balance results for C18-solid phase extraction and ultrafiltration (% DOC retained from DOC (<0.2 µm) after fractionation). CF (concentration factor) = ratio of the initial sample volume to the volume of retentate at the end of filtration. % aliphatic = 0-110 ppm and % aromatic = 110-160 ppm from solid-state $^{13}$C NMR analyses. Solid phase extracted = SPE, nd = not determined.

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<td>17</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R4 MC</td>
<td>39</td>
<td>140</td>
<td>SPE</td>
<td>140</td>
<td>23</td>
<td>70</td>
<td>17</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R4 P</td>
<td>41</td>
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<td>SPE</td>
<td>160</td>
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<td>71</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R5 MC</td>
<td>34</td>
<td>145</td>
<td>SPE</td>
<td>145</td>
<td>29</td>
<td>74</td>
<td>14</td>
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</tr>
<tr>
<td>August 1999</td>
<td>flood</td>
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<td>76</td>
<td>120</td>
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<td>55</td>
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<td>61</td>
<td>25</td>
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<td>73</td>
<td>150</td>
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<td>29</td>
<td>11</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>October 1999</td>
<td>medium-flow</td>
<td>R2 MC</td>
<td>47</td>
<td>177</td>
<td>SPE</td>
<td>177</td>
<td>28</td>
<td>66</td>
<td>19</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R4 MC</td>
<td>51</td>
<td>170</td>
<td>SPE</td>
<td>170</td>
<td>22</td>
<td>66</td>
<td>19</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R4 P</td>
<td>35</td>
<td>140</td>
<td>SPE</td>
<td>140</td>
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<td>69</td>
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<tr>
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<td></td>
<td>R5 MC</td>
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<td>185</td>
<td>42</td>
<td>65</td>
<td>21</td>
<td>3.1</td>
</tr>
<tr>
<td>November 1999</td>
<td>high-flow</td>
<td>R4 MC</td>
<td>67</td>
<td>160</td>
<td>ultrafiltered</td>
<td>64</td>
<td>13</td>
<td>62</td>
<td>29</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R4 P</td>
<td>48</td>
<td>122</td>
<td>ultrafiltered</td>
<td>56</td>
<td>8</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R5 MC</td>
<td>50</td>
<td>165</td>
<td>ultrafiltered</td>
<td>83</td>
<td>10</td>
<td>54</td>
<td>25</td>
<td>2.2</td>
</tr>
<tr>
<td>April 2000</td>
<td>high-flow</td>
<td>R2 MC</td>
<td>85</td>
<td>80</td>
<td>SPE</td>
<td>80</td>
<td>31</td>
<td>67</td>
<td>18</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R4 MC</td>
<td>95</td>
<td>70</td>
<td>SPE</td>
<td>70</td>
<td>43</td>
<td>68</td>
<td>18</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R4 P</td>
<td>56</td>
<td>70</td>
<td>SPE</td>
<td>70</td>
<td>54</td>
<td>65</td>
<td>20</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R5 MC</td>
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<td>80</td>
<td>SPE</td>
<td>80</td>
<td>48</td>
<td>79</td>
<td>18</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R2 MC</td>
<td>85</td>
<td>70</td>
<td>ultrafiltered</td>
<td>50</td>
<td>24</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R4 MC</td>
<td>95</td>
<td>70</td>
<td>ultrafiltered</td>
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<td>77</td>
<td>16</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R5 MC</td>
<td>83</td>
<td>80</td>
<td>ultrafiltered</td>
<td>40</td>
<td>22</td>
<td>55</td>
<td>29</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* R2, headwater floodplain; R4, major floodplain; R5, lower floodplain; MC, main channel; P, isolated pool in major floodplain
Table 3.2
Compounds identified in the thermochozymes of solid phase extracted DOM samples R4MC and R5MC.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Hydrophobic DOM compound</th>
<th>Peak</th>
<th>Hydrophobic DOM compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TMAH products of uncertain origin</td>
<td></td>
<td>TMAH products of uncertain origin</td>
</tr>
<tr>
<td>1</td>
<td>C8 alcohol branched</td>
<td>1</td>
<td>alcohol branched</td>
</tr>
<tr>
<td>2</td>
<td>butanedioic acid dimethyl ester</td>
<td>2</td>
<td>4-oxo pentanoic acid methyl ester</td>
</tr>
<tr>
<td>3</td>
<td>methyl, butanedioic acid dimethyl ester</td>
<td>3</td>
<td>butanedioic acid dimethyl ester</td>
</tr>
<tr>
<td>4</td>
<td>benzoic acid methyl ester</td>
<td>4</td>
<td>methyl, butanedioic acid dimethyl ester</td>
</tr>
<tr>
<td>5</td>
<td>pentanedioic acid dimethyl ester</td>
<td>5</td>
<td>2-methoxyphenol</td>
</tr>
<tr>
<td>6</td>
<td>1,4-dimethoxybenzene</td>
<td>6</td>
<td>benzoic acid methyl ester</td>
</tr>
<tr>
<td>7</td>
<td>2-methyl, pentanedioic acid dimethyl ester</td>
<td>7</td>
<td>pentanedioic acid dimethyl ester</td>
</tr>
<tr>
<td>8</td>
<td>4-chlorobenzoic acid methyl ester</td>
<td>8</td>
<td>1,4-dimethoxybenzene</td>
</tr>
<tr>
<td>9</td>
<td>hexanedioic acid dimethyl ester</td>
<td>9</td>
<td>2-methyl pentanedioic acid dimethyl ester</td>
</tr>
<tr>
<td>10</td>
<td>phthalic anhydride</td>
<td>10</td>
<td>1,4-dimethoxy-2-methyl benzene</td>
</tr>
<tr>
<td>11</td>
<td>dimethyl phthalate</td>
<td>11</td>
<td>hexanedioic acid dimethyl ester</td>
</tr>
<tr>
<td>12</td>
<td>chloro-methoxy-benzoic acid methyl ester</td>
<td>12</td>
<td>TMAH</td>
</tr>
<tr>
<td>13</td>
<td>honanedioic acid dimethyl ester</td>
<td>13</td>
<td>phthalic anhydride</td>
</tr>
<tr>
<td>14</td>
<td>3,4-dimethoxybenzoic acid methyl ester</td>
<td>14</td>
<td>1,2,4-trimethoxybenzene (isomer of S2)</td>
</tr>
<tr>
<td>15</td>
<td>methyl, 6,8-dodecadienyl ether</td>
<td>15</td>
<td>1,3,5-trimethoxybenzene (derived from cutane)</td>
</tr>
<tr>
<td>16</td>
<td>octadecon (C18)</td>
<td>16</td>
<td>methyl, 4,6-decadienyl ether</td>
</tr>
<tr>
<td>17</td>
<td>hexadecanol</td>
<td>17</td>
<td>dimethyl phthalate</td>
</tr>
<tr>
<td>18</td>
<td>eicosane (internal standard)</td>
<td>18</td>
<td>1-chloro dodecane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>1,4-benzene dicarboxylic acid dimethyl ester</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>3,4-dimethoxy benzoic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>methyl, 6,8-dodecadienyl ether</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>3,4-dimethoxybenzenoacetic acid methyl ester</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23</td>
<td>1-chloro-tetradecane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>eicosane (internal standard)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polysaccharide derived TMAH products</th>
<th>Polysaccharide derived TMAH products</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1 2-methyl-2-cyclopenten-1-one</td>
<td>PS1 2-ethyl-3-methoxy-2-cyclopentenone</td>
</tr>
<tr>
<td>PS3 3,4-dimethyl-2-cyclopent-1-one</td>
<td></td>
</tr>
<tr>
<td>PS6 2-ethyl-3-methoxy-2-cyclopentenone</td>
<td></td>
</tr>
<tr>
<td>PS7 1-(3,4,5-trimethoxy phenyl) ethanol</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lignin derived TMAH products</th>
<th>Lignin derived TMAH products</th>
</tr>
</thead>
<tbody>
<tr>
<td>syringyl derived compounds</td>
<td>syringyl derived compounds</td>
</tr>
<tr>
<td>S1 methoxy methyl benzene</td>
<td>methoxy methyl benzene</td>
</tr>
<tr>
<td>S2 3,4,5-trimethoxybenzoic acid methyl ester</td>
<td>1,2,3-trimethoxy benzene</td>
</tr>
<tr>
<td>S3 3,4,5-trimethoxybenzoic acid methyl ester</td>
<td>3,4,5-trimethoxybenzoic acid methyl ester</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>guaiacyl derived compounds</th>
<th>guaiacyl derived compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 1,2-dimethoxy benzene</td>
<td>1,2-dimethoxy benzene</td>
</tr>
<tr>
<td>G2 3,4-dimethoxy benzaldehyde</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>p-hydrophenyl derived compounds</th>
<th>4-methoxybenzoic acid methyl ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fatty acid methyl esters (FAMES)</th>
<th>Fatty acid methyl esters (FAMES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:0 FAME octanoic acid methyl ester</td>
<td>8:0 FAME octanoic acid methyl ester</td>
</tr>
<tr>
<td>10:0 FAME decanoic acid methyl ester</td>
<td>14:0 FAME tetradecanoic acid methyl ester</td>
</tr>
<tr>
<td>15:0 FAME pentadecanoic acid methyl ester</td>
<td>iso 15:0 FAME 2-methyl, tetradecanoic acid methyl ester</td>
</tr>
<tr>
<td>16:1 FAME hexadecanoic acid methyl ester</td>
<td>anteiso 15:1 FAME 3-methyl, tetradecanoic acid methyl ester</td>
</tr>
<tr>
<td>18:1 FAME octadecenoic acid methyl ester</td>
<td>15:0 FAME pentadecanoic acid methyl ester</td>
</tr>
<tr>
<td>18.0 FAME octadecanoic acid methyl ester</td>
<td>16:1 FAME hexadecanoic acid methyl ester</td>
</tr>
<tr>
<td></td>
<td>16:0 FAME hexadecanoic acid methyl ester</td>
</tr>
<tr>
<td></td>
<td>18:1 FAME octadecanoic acid methyl ester</td>
</tr>
<tr>
<td></td>
<td>18:0 FAME octadecanoic acid methyl ester</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N containing compounds</th>
<th>N containing compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1 unknown heterocyclic N compound (m/z 42,127)</td>
<td>N1 3-ethyl-1,3-dimethyl-2,5-pyrrolidinedione</td>
</tr>
<tr>
<td>N2 3-ethyl-1,3-dimethyl-2,5-pyrrolidinedione</td>
<td>N2 2-methyl-(1H)-isoindole-1,3 (2H) dione</td>
</tr>
<tr>
<td>N3 2-methyl-(1H)-isoindole-1,3 (2H) dione</td>
<td>N3 dimethoxy acetylperitone</td>
</tr>
</tbody>
</table>

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Table 3.3
Compounds identified in the thermochemozymes of the ultrafiltered DOM samples from R4MC and R5MC.

<table>
<thead>
<tr>
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<th>HMW DOM compound</th>
<th>Peak</th>
<th>HMW DOM compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O-methyl, sulfone</td>
<td>3</td>
<td>butanedioic acid dimethyl ester</td>
</tr>
<tr>
<td>2</td>
<td>2,2-dimethyl-2-cyclopentene-1-one</td>
<td>6</td>
<td>benzoic acid methyl ester</td>
</tr>
<tr>
<td>3</td>
<td>butanedioic acid dimethyl ester</td>
<td>7</td>
<td>pentanedioic acid dimethyl ester</td>
</tr>
<tr>
<td>4</td>
<td>methyl, butanedioic acid dimethyl ester</td>
<td>24</td>
<td>eicosane (internal standard)</td>
</tr>
<tr>
<td>5</td>
<td>benzoic acid methyl ester</td>
<td>6</td>
<td>pentanedioic acid dimethyl ester</td>
</tr>
<tr>
<td>7</td>
<td>2-methyl, pentanedioic acid dimethyl ester</td>
<td>8</td>
<td>1,4-dimethoxybenzene</td>
</tr>
<tr>
<td>9</td>
<td>hexanedioic acid dimethyl ester</td>
<td>10</td>
<td>TMAH</td>
</tr>
<tr>
<td>11</td>
<td>3-methoxybenzoic acid methyl ester</td>
<td>12</td>
<td>4-methyl-3-methoxybenzoic acid methyl ester</td>
</tr>
<tr>
<td>13</td>
<td>1,2,3-trimethoxy-5-methyl benzene</td>
<td>14</td>
<td>2-methoxybenzene propanoic acid methyl ester</td>
</tr>
<tr>
<td>15</td>
<td>3,4 dimethoxybenzoic acid methyl ester</td>
<td>16</td>
<td>eicosane (internal standard)</td>
</tr>
</tbody>
</table>

**Polyasaccharide derived TMAH products**

<table>
<thead>
<tr>
<th>Peak</th>
<th>HMW DOM compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1</td>
<td>2-methyl-2-cyclopenten-1-one</td>
</tr>
<tr>
<td>PS2</td>
<td>methyl, cycloheptene</td>
</tr>
<tr>
<td>PS3</td>
<td>3,4-dimethyl-2-cyclopenten-1-one</td>
</tr>
<tr>
<td>PS4</td>
<td>methyl, 2-furate</td>
</tr>
<tr>
<td>PS5</td>
<td>3,4-dimethyl-2-cyclopenten-1-one</td>
</tr>
<tr>
<td>PS6</td>
<td>2-ethyl-3-methoxy-2-cyclopentene</td>
</tr>
</tbody>
</table>

**Lignin derived TMAH products**

<table>
<thead>
<tr>
<th>Syringyl derived compounds</th>
<th>Guaiacyl derived compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>methoxymethylbenzene</td>
<td>1,2,3-trimethoxybenzene</td>
</tr>
<tr>
<td>S1</td>
<td>G1</td>
</tr>
</tbody>
</table>

**Fatty acid methyl esters (FAMES)**

<table>
<thead>
<tr>
<th>FAME</th>
<th>HMW DOM compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:0 FAME</td>
<td>heptanoic acid methyl ester</td>
</tr>
<tr>
<td>8:0 FAME</td>
<td>octanoic acid methyl ester</td>
</tr>
<tr>
<td>9:0 FAME</td>
<td>nonanoic acid methyl ester</td>
</tr>
<tr>
<td>10:0 FAME</td>
<td>decanoic acid methyl ester</td>
</tr>
<tr>
<td>14:0 FAME</td>
<td>tetradecanoic acid methyl ester</td>
</tr>
<tr>
<td>16:0 FAME</td>
<td>hexadecanoic acid methyl ester</td>
</tr>
<tr>
<td>18:1 FAME</td>
<td>octadecenoic acid methyl ester</td>
</tr>
</tbody>
</table>

**N containing compounds**

<table>
<thead>
<tr>
<th>Peak</th>
<th>HMW DOM compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>2,2,4,4-tetramethyl-1,3-cyclopentane dione</td>
</tr>
<tr>
<td>N2</td>
<td>1-methyl-2,5-pyrrolidinedione</td>
</tr>
<tr>
<td>N3</td>
<td>unknown heterocyclic N compound (m/z 42, 127)</td>
</tr>
<tr>
<td>N4</td>
<td>3-ethyl-1,3-dimethyl-2,5-pyrrolidinedione</td>
</tr>
<tr>
<td>N5</td>
<td>2-methyl-(2H)-isoindole-1,3 (2H) dione</td>
</tr>
</tbody>
</table>
CHAPTER 4

Phototransformation of riverine dissolved organic matter (DOM) in the presence of abundant iron: Effect on DOM bioavailability

KAISER, E. AND SULZBERGER, B.

We have conducted studies with high- (HMW) and low-molecular weight (LMW), hydrophobic and hydrophilic DOM from an oligotrophic, alpine river, the Tagliamento River (Italy), to assess the effect of light on DOM utilization by natural riverine bacterioplankton. Immediately after exposure of the DOM fractions to simulated or natural sunlight, the short-term (1 hour) bacterial utilization of all DOM fractions decreased by up to 80%, as compared to uptake of the corresponding non-irradiated fractions. The addition of scavengers past irradiation suggests that reactive oxygen species (ROS) caused this inhibition of bacterial growth. After long-term growth of bacteria on non- and irradiated DOM, uptake was unchanged for irradiated HMW DOM, considerably lower for irradiated LMW and hydrophilic DOM, and much higher for irradiated hydrophobic DOM, as compared to the non-irradiated incubations. These results suggest that phototransformations of HMW, LMW, hydrophobic and hydrophilic DOM result in contrasting effects on the bioavailability of these different DOM fractions. Size exclusion chromatography showed that bacteria preferably utilized the larger molecular sizes of all non-irradiated fractions. Light induced no significant shifts in the apparent molecular weight distribution of all four DOM fractions. However, the highly bioavailable LMW DOM (especially the portion sizing ~5 kD) was no longer taken up after irradiation. From this study we conclude that in the Tagliamento River, light overall leads to a strong decrease of microbial DOM transformation during hydrological transport.


Introduction

DOM represents a major reactive reservoir of organic carbon on Earth (Schlesinger and Melack 1981; Hedges et al. 1992) and an essential energy resource for all microbial processes (Wetzel 1992). River systems are key to the global carbon cycling, because they receive, produce, transport, and transform organic material, and therefore, integrate terrestrial and marine environments (Berner 1989; Meybeck 1981; Hedges et al. 1992; 1997; Raymond and Bauer 2001).

In the last two decades the importance of photochemical transformations for the cycling of organic carbon in aquatic systems has been recognized and intensively studied (Kieber et al. 1989; Mopper et al. 1991; Benner and Biddanda 1998). Some studies described the net effect of solar radiation on bacteria-DOM interactions. They showed that photocleavage and photooxidation of HMW DOM led to a release of readily bioavailable LMW compounds, which indirectly stimulated bacterialplankton activity and thus enhanced the turnover of DOM (Kieber et al. 1989; Lindell et al. 1995; Wetzel et al. 1995). Other work proved that light led to contrasting effects on DOM, transforming bioavailable into biorecalcitrant compounds and vice versa (Tranvik and Kokalj 1998; Benner and Biddanda 1998; Obernosterer et al. 1999). In surface marine waters and humic lakes, sunlight was also found to abiotically remineralize a large portion of DOM to carbon monoxide/carbon dioxide and inorganic nutrients such as phosphate and ammonium (Cotner and Heath 1990; Miller and Zepp 1995; Bushaw et al. 1996; Johannsssen and Miller 2000). Some studies characterized light-induced changes in DOM bulk chemical composition using $^{13}$C NMR (Wetzel et al. 1995; Clair and Sayer 1997). Opsahl and Benner (1998) investigated light-induced changes in the dissolved lignin composition, with lignin being an important biomarker for terrestrial organic biomass transported dominantly by rivers to the marine environment. Although the literature provides ample information on DOM phototransformations in lake and surface ocean environments, we hitherto lack knowledge on the impact of sunlight on riverine carbon cycling (Amon and Benner 1996).

Rivers carry high amounts of particulate and colloidal iron, which they receive through weathering of minerals (e.g., biotite) in the catchment area and oxidative precipitation of Fe(II). In iron-rich surface waters light-induced redox cycling of iron and
DOM photooxidation are strongly coupled. Iron can catalyze DOM photooxidation via ligand-to-metal charge transfer reactions of Fe(III)-DOM complexes, and through DOM oxidation by the hydroxyl radical (OH\(^-\)) formed in the Fenton reaction (Miles and Brezonik, 1981; Voelker et al. 1997). The direct photooxidation of DOM (i.e., electron transfer from triplet-excited functional groups of DOM to ground-state molecular oxygen) yields superoxide (\(O_2^-\)), which reacts to \(H_2O_2\) by (iron-catalyzed) dismutation (Bielski et al. 1985; Haag and Mill 1990; Blough and Zepp 1995; Voelker et al. 1997). In addition, oxidation of Fe(II) by oxygen and subsequent reactions also produce the transient species \(O_2^-\), \(H_2O_2\), and \(OH^-\) (e.g., Moffet and Zika 1987; Emmenegger et al. 1998). Major sinks of \(O_2^-\) are oxidation and reduction of iron and copper species, and reaction with DOM (Goldstone and Voelker 2000). In addition to \(H_2O_2\), long-living organic peroxides (ROOH) also are produced in photochemical and subsequent thermal reactions of DOM (von Sonntag and Schuchmann 1997).

Reactive oxygen species, such as \(O_2^-\), \(H_2O_2\), and \(HO^-\), organic peroxyl radicals (RO\(_2^-\)), and organic peroxides (ROOH), resulting from photochemical and subsequent thermal reactions of DOM and iron, have been shown to severely damage the cell structure and physiology of aquatic microorganisms (Fridovich 1986; Asada and Takahashi 1987). For example, reactive species initiate the oxidation of chlorophyll, the peroxidation of lipids, and inhibit carbon fixation and the motility of dinoflagellates. However, we still know little about their effect on bacterial activity. As a defense against active forms of oxygen, microorganisms have developed various biochemical antioxidants, quenchers, and scavengers (Chow 1988; Sies 1985), such as tocopherols, carotenoids, ascorbate, urate, and enzymes.

In the Tagliamento River, light may significantly penetrate the water column due to low turbidity and dominantly shallow depth of the water bodies and be absorbed by DOM or iron-DOM complexes. Therefore, one important question is how well cells can utilize DOM that has been exposed to sunlight in this alpine river system. To increase our understanding of the overall effect of light on bacterial DOM utilization, our approach was to study different fractions of DOM. Over more than one year, we bimonthly collected water in a longitudinal and lateral direction from the Tagliamento River (Fig. 1). We size- and chemically fractionated these water samples to gain HMW and LMW as
well as hydrophobic and hydrophilic DOM. These four fractions were used for photochemical and bioassay experiments. The purpose of our research was (i) to determine the short- and long-term effects of irradiation of DOM fractions on their utilization by natural bacterioplankton, and (ii) to relate light-induced changes of the bioavailability of DOM fractions to changes of their chemical composition.

Figure 4.1
Catchment site of the Tagliamento River with important sampling stations marked by large grey patches: Main channel in headwater floodplain (R2MC), in major floodplain (R4MC), and in transition floodplain (R5MC), and isolated pool in major floodplain (R4P). Find the most important tributary streams, the Arzino, But, Degano, and Fella in the catchment area. The little inset shows that the river is located northeast Italy, Europe.
Materials and methods

Study area and sample collection

The whole river corridor of the Tagliamento River has been extensively studied since 1997 (Ward et al. 1999b; Arscott et al. 2000; Gurnell et al. 2000; Nat van der et al. 2002). The river is classified as the last large natural river in Europe (northeast Italy) (Ward et al. 1999b) extending over 170 km and flowing unrestrained from the alpine headwater reaches to the northern Adriatic Sea (Fig. 4.1). Its highly complex channel morphology is characterized by constrained, braided, and meandering reaches formed by a dynamic hydrological regime. The Tagliamento River experiences two major floods within a year (spring and fall), facilitating the deposition and mobilization of materials and leading to major changes in organic matter input and transport.

The water samples for DOM fractionation were seasonally collected from the main channels (MC) in the island-braided headwater floodplain (R2), in the island-braided lower and major floodplain (R4), and in the braided-to-meandering transition floodplain (R5) as well as from one isolated pool (P) in R4 (Fig. 4.1). Field sampling took place during March, May, July, August, October, November 1999, and April, July, and August 2000. During sampling the water levels varied from low- to high-flow conditions (Table 4.1). Over the year, the temperatures of the main channel river water were 10.9 ± 2.5°C, the pH’s were 8.13 ± 0.14, and the alkalinites were 2.8 ± 0.5 mmol l⁻¹. Water samples (~200 l per sampling station) for DOM fractionation were collected with clean 50 L high-density polyethylene (HDPE) carboys and transported to the field laboratory located close to the Tagliamento River in R4. Immediately following collection, water samples were passed through muffled GF/F- and 0.2 µm Durapore filters (142 mm diameter, Millipore) and stored in clean 50 l carboys.

DOM fractionation

DOM was either chemically or size fractionated, except in April 2000, when both methods were employed in parallel. For chemically fractionating DOM into hydrophobic- and hydrophilic-type compounds, a Mega Bond Elute C18 column (C18 loaded silica, 60CC, Varian) was used after acidification with 32% hydrochloric acid (Suprapur) to pH 2.8 (Louchouarn et al. 2001). Before fractionation, the water was acidified. Flow rates
range from 3 - 5 L h\(^{-1}\). Of the total DOC (DOC <0.2 \(\mu\)m), 13 - 54% was recovered as a sorbed hydrophobic fraction (Table 1), being comparable to other literature values (Mills and Quinn 1981; Amador et al. 1990). A Filtron tangential flow ultrafiltration system with a polyethersulfone membrane (1 kDa nominal weight cutoff) was used for fractionating bulk DOM into pseudo HMW and LMW portions, following the protocol of Benner et al. (1997). Operating pressures were 137.9 - 151.7 kPa at the inlet and 68.9 - 89.6 kPa at the outlet. Filtration rates range between 6 - 8 l h\(^{-1}\) using one 0.46 m\(^2\) cassette filter (Centrasette, Filtron). The water temperature ranged from 20 - 22\(^\circ\)C during ultrafiltration. Recovery was 8 - 29% of the total DOC (Table 4.1). For every sample, a carbon mass balance was established to determine whether carbon was lost or gained during fractionation. Mass balance calculations revealed recoveries of 99 – 144% for ultrafiltration and 100 – 139% for C18 solid phase extraction. The percentage of DOC recovered after fractionation from bulk DOC was calculated as follows: \%
DOC retained
= \frac{100 \times (DOC_{\text{retentate}} + DOC_{\text{permeate}})}{DOC_{\text{<0.2\(\mu\)m}}} - 1\%

From fractionation, samples were stored at 4\(^\circ\)C in the dark and immediately transported back to the laboratory in Switzerland (EAWAG). DOM adsorbed onto the C18 phase was eluted by gentle vacuum-filtration using high-purity methanol (Merck). The methanol was removed from DOM by roto-evaporation and freeze-drying. Next, the extract was redissolved in MQ-UV water (Millipore). For further photochemical experiments, hydrophobic and HMW DOM were re-diluted with MQ-UV water to reach final DOC concentrations of ~100 \(\mu\)mol l\(^{-1}\) C, which was in the range of TOC concentrations measured from raw river water.
Table 4.1
Sample descriptions, dissolved organic carbon (DOC) and dissolved iron (DI) concentrations in the individual DOM fractions, DI as % of bulk DI measured in bulk DOM (<0.2 µm), Fe(II) steady-state concentrations ([Fe(II)]\textsubscript{ss}), [Fe(II)]\textsubscript{ss} as % of DI, and net H\textsubscript{2}O\textsubscript{2} formation. Data represent averages (± standard error) of seasonal samples (n = 3 - 5). nd = not determined. DOC concentrations of HMW and hydrophobic DOM are corrected by the concentrator factor calculated from ultrafiltration and C18 solid phase extraction.

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>DOM fraction</th>
<th>DOC (µmol l\textsuperscript{-1})</th>
<th>DI (nmol l\textsuperscript{-1})</th>
<th>DI as % of bulk DI</th>
<th>[Fe(II)]\textsubscript{ss} (nmol l\textsuperscript{-1} / 1mg C l\textsuperscript{-1})</th>
<th>[Fe(II)]\textsubscript{ss} (% of DI)</th>
<th>Net H\textsubscript{2}O\textsubscript{2} formation (nmol l\textsuperscript{-1} / 4 h\textsuperscript{-1}) / (1mg C l\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2MC</td>
<td>hydrophobic</td>
<td>17 ± 5</td>
<td>5.0 ± 3.0</td>
<td>30 ± 14</td>
<td>3.0 ± 0.3</td>
<td>295 ± 9</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>hydrophilic</td>
<td>45 ± 7</td>
<td>10.5 ± 2.2</td>
<td>83 ± 31</td>
<td>7.1 ± 6.2</td>
<td>27 ± 24</td>
<td>257 ± 90</td>
</tr>
<tr>
<td></td>
<td>HMW</td>
<td>13 ± 3</td>
<td>2.6 ± 0.8</td>
<td>13 ± 1</td>
<td>3.4 ± 1.6</td>
<td>20 ± 11</td>
<td>6 ± 4</td>
</tr>
<tr>
<td></td>
<td>LMW</td>
<td>76 ± 3</td>
<td>22.2 ± 15.8</td>
<td>45 ± 10</td>
<td>0.6 ± 0.3</td>
<td>1 ± 1</td>
<td>248 ± 9</td>
</tr>
<tr>
<td>R4MC</td>
<td>hydrophobic</td>
<td>19 ± 7</td>
<td>14.0 ± 6.0</td>
<td>44 ± 14</td>
<td>0.7 ± 0.6</td>
<td>2 ± 1</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>hydrophilic</td>
<td>48 ± 8</td>
<td>35.9 ± 9.6</td>
<td>49 ± 20</td>
<td>1.2 ± 0.8</td>
<td>4 ± 2</td>
<td>282 ± 18</td>
</tr>
<tr>
<td></td>
<td>HMW</td>
<td>20 ± 4</td>
<td>12.8 ± 4.6</td>
<td>38 ± 14</td>
<td>2.1 ± 0.9</td>
<td>4 ± 3</td>
<td>83 ± 33</td>
</tr>
<tr>
<td></td>
<td>LMW</td>
<td>91 ± 14</td>
<td>13.3 ± 4.5</td>
<td>23 ± 2</td>
<td>0.3 ± 0.2</td>
<td>4 ± 2</td>
<td>201 ± 65</td>
</tr>
<tr>
<td>R4P</td>
<td>hydrophobic</td>
<td>18 ± 5</td>
<td>12.0 ± 11.0</td>
<td>84 ± 3</td>
<td>0.7 ± 0.4</td>
<td>3 ± 1</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>hydrophilic</td>
<td>30 ± 3</td>
<td>15 ± 5</td>
<td>83 ± 57</td>
<td>4.9 ± 2.2</td>
<td>12 ± 6</td>
<td>431 ± 147</td>
</tr>
<tr>
<td></td>
<td>HMW</td>
<td>10 ± 3</td>
<td>10.6 ± 6.9</td>
<td>28 ± 10</td>
<td>3.2 ± 1.4</td>
<td>12 ± 7</td>
<td>88 ± 56</td>
</tr>
<tr>
<td></td>
<td>LMW</td>
<td>65 ± 14</td>
<td>9.6 ± 2.8</td>
<td>76 ± 64</td>
<td>0.5 ± 0.2</td>
<td>4 ± 2</td>
<td>293 ± 73</td>
</tr>
<tr>
<td>R5MC</td>
<td>hydrophobic</td>
<td>19 ± 8</td>
<td>6.0 ± 4.0</td>
<td>32 ± 9</td>
<td>nd</td>
<td>nd</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>hydrophilic</td>
<td>31 ± 4</td>
<td>12.1 ± 3.4</td>
<td>59 ± 19</td>
<td>12.7 ± 10</td>
<td>24 ± 17</td>
<td>315 ± 102</td>
</tr>
<tr>
<td></td>
<td>HMW</td>
<td>12 ± 3</td>
<td>4.9 ± 1.1</td>
<td>40 ± 7</td>
<td>2.5 ± 0.6</td>
<td>6 ± 1</td>
<td>101 ± 50</td>
</tr>
<tr>
<td></td>
<td>LMW</td>
<td>64 ± 11</td>
<td>16.2 ± 9.4</td>
<td>104 ± 77</td>
<td>0.4 ± 0.3</td>
<td>2 ± 1</td>
<td>133 ± 9</td>
</tr>
</tbody>
</table>

* R2, headwater floodplain; R4, major floodplain; R5, lower floodplain; MC, main channel; P, isolated pool in major floodplain

**TOC and DOC measurements**
Water samples (20 ml) were collected directly after fractionation for ultrafiltered samples and after freeze-drying and redissolution in MQ-UV water for solid phase extracted
DOM. The samples were filled in acid-rinsed, pre-combusted 40 ml EPA glass vials, sealed with Teflon-lined caps, and stored frozen until analysis. Total organic carbon (TOC) and DOC concentrations were determined by high temperature catalytic oxidation with a Shimadzu 5050A analyzer (Benner et al. 1993; 1997).

**Size exclusion chromatography (SEC)**

Water samples for SEC were collected in muffled 10 ml ampoules or acid-rinsed, muffled 40 ml EPA glass vials (Wheaton), sealed with Teflon-lined caps, and stored frozen until analysis. We used gel permeation chromatography (TSKHW50S) with UV-absorbance (at 254 nm) and DOC detection to characterize the apparent molecular weight distribution of the DOM fractions prior to and after irradiation (Mueller et al. 2000). The mass-selective detection of DOC is based on the absorption of infrared light by CO$_2$ from photooxidized DOC. The mobile phase was composed of “carbon-free” water (UV-photo-oxidized MQ-UV water, Millipore), 3.6 mmol L$^{-1}$ sodiumhydrogenphosphate, and 18.4 mmol L$^{-1}$ potassiumhydrogenphosphate (pH 6.6). Potassiumhydrogenphosphate was also used for calibration. A variety of organic model substances were employed to describe the size exclusion of different molecular weight ranges between 200 to 0.1 kDalton (Huber and Frimmel 1992). Humic substances, LMW organic acids, polysaccharides, proteins, and amphiphilic and hydrophobic organic substances were used for qualitative interpretations of chromatograms obtained with the DOM fractions. We calculated the apparent molecular size distribution after Perminkova et al. (1998).

**Total and dissolved iron (Fe(II), Fe(III)) measurements**

Before and after DOM fractionation all water samples were collected in clean acid-rinsed 10 ml PP-tubes (Greiner). After acidification to pH~2 (with cold-distilled 80% HNO$_3$, Suprapur) all samples were stored at 4ºC for total and dissolved Fe measurements. Iron was analyzed using a Perkin-Elmer 5100 GF-AAS with a THGA graphite tube atomizer. Samples containing less than 20 nmol Fe L$^{-1}$ were pre-concentrated three times by injecting 20 µl of the sample and drying for 50 s at 130ºC. The detection limit was 3 nmol L$^{-1}$ for the pre-concentrated samples. For every sample an iron mass balance was established to determine if iron was gained or lost during fractionation. The dissolved
Iron concentrations (DI) were measured of 0.2 µm filtered water (Table 4.1) and of all different DOM fractions (data not shown). The percentage of DI recovered after fractionation from bulk DI (<0.2 µm) was calculated as follows: % DI recovered = 100 × (DI\_retentate + DI\_permeate) / (DI\_<0.2\mu m)\(^{-1}\). The DI\_retentate concentrations were determined by using the concentration factors from DOC mass balances. The DI mass balance calculations revealed recoveries of 45 – 180% for ultrafiltration and 89 – 160% for C18 solid phase extraction.

Iron(II) was measured during and past irradiation of the DOM fractions (Fig. 4.2) by using a flow injection analysis system with a luminol-based chemiluminescence detection (FeLume) (King et al. 1995; Emmenegger et al. 2001). Fe(II) samples were introduced into the flow cell and mixed with an ammonia-buffered luminol reagent. At pH 9.8, Fe(II) is oxidized by oxygen on a millisecond-time scale catalyzing the oxidation of luminol and producing blue chemiluminescence light.

**Simulated solar radiation (~1 kWm\(^{-2}\))**

<table>
<thead>
<tr>
<th>4-h exposure</th>
<th>10-h exposure</th>
<th>Solar radiation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>prior to irradiation</strong></td>
<td><strong>prior to irradiation</strong></td>
<td><strong>prior to irradiation</strong></td>
</tr>
<tr>
<td>TOC/DOC and H(_2)O(_2) concentrations</td>
<td>TOC/DOC concentrations</td>
<td>TOC/DOC concentrations</td>
</tr>
<tr>
<td>SEC</td>
<td>SEC</td>
<td>pH, SEC</td>
</tr>
<tr>
<td>bacterial activity</td>
<td>bacterial activity</td>
<td>bacterial activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>bioassay experiment</strong></td>
</tr>
<tr>
<td><strong>after irradiation</strong></td>
<td><strong>after irradiation</strong></td>
<td><strong>after irradiation</strong></td>
</tr>
<tr>
<td>TOC/DOC and H(_2)O(_2) concentrations</td>
<td>TOC/DOC concentrations</td>
<td>TOC/DOC concentrations</td>
</tr>
<tr>
<td>SEC, radical scavenger</td>
<td>SEC, bacterial activity</td>
<td>pH, SEC</td>
</tr>
<tr>
<td>bacterial activity</td>
<td>bacterial activity</td>
<td>bacterial activity</td>
</tr>
<tr>
<td></td>
<td><strong>at start</strong></td>
<td><strong>at end</strong></td>
</tr>
<tr>
<td>TOC/DOC concentrations, SEC, bacterial activity</td>
<td>TOC/DOC concentrations</td>
<td>TOC/DOC concentrations</td>
</tr>
<tr>
<td></td>
<td><strong>frequent sampling for bacterial activity</strong></td>
<td><strong>at end</strong></td>
</tr>
<tr>
<td></td>
<td><strong>at end</strong></td>
<td></td>
</tr>
</tbody>
</table>
Hydrogen peroxide (H$_2$O$_2$) measurements
Prior to and at the end of the irradiation the DOM fractions were sampled in duplicates and immediately analyzed. For measuring H$_2$O$_2$ we used the method by Miller (1988) and Emmenegger et al. (2001) and utilized a Perkin-Elmer LS-3 fluorescence spectrophotometer. We also found that potential light-induced production of organic peroxides was not influencing the H$_2$O$_2$ fluorescence measurements (data not shown).

Bacterial abundance and biomass production
Samples (10 ml) for microscopy were preserved with a filtered borax-buffered formaldehyde solution (5% final concentration) and stored refrigerated in the dark. Bacterial abundance was determined within two weeks of sample collection by epifluorescence microscopy (Olympus BX50, 1,000 magnification) of 4’,6-diamidino-2-phenylindole (DAPI)-stained cells (Porter and Feig 1980). In the laboratory and field experiments, bacterial biomass production was estimated from protein synthesis by adding [$^3$H]leucine (20 nmol l$^{-1}$ final concentration) to 5 ml samples and incubating them in the dark at 20ºC for 60 minutes (Kirchman 1986). All samples were measured in triplicates with two formaline-killed (2% final concentration) blanks. After incubation, the samples were filtered onto cellulosenitrate filters (GSWP, 0.2 µm pore-size, 25 mm diameter, Millipore) and rinsed twice with 5 ml of chilled 5% trichloroacetic acid. The filters were placed in scintillation vials with 10 ml of scintillation cocktail (Insta-Gel, Packard) added. Radioactivity was assessed with a liquid scintillation counter (Packard Tri-Carb 2000) by external standard ratio technique. We used the factor 3.1 µg C nmol$^{-1}$ leucine for converting leucine incorporation into the bacterial biomass (Simon and Azam 1989). In the following text, bacterial biomass production, which was normalized by bacterial abundance of the individual sample, is used to express bacterial DOM utilization (or bacterial DOM uptake).

Irradiation experiments
In the laboratory, all DOM fractions were irradiated for 4 hours with a 1000 W Xe-lamp (OSRAM, PTI) to simulate a full solar day (Fig. 4.2). The light intensity was approximately 1 kW m$^{-2}$, as determined by ferrioxalate actinometry (Hatchard and
Parker, 1956). The light was focused by two Pyrex lenses (50% cutoff at 335 nm) and a mirror onto a water-jacketed reactor with a quartz bottom window. A more detailed description of the experimental set-up has been published by Siffert and Sulzberger (1991). During exposure, the sample was continuously stirred, the temperature was kept constant at 22 °C, and the pH was determined with an Orion Ross electrode and an Orion meter calibrated with NBS buffers. Prior and after irradiation we sampled for TOC, DOC, SEC, H2O2, and bacterial DOM utilization (Fig. 4.2). To determine bacterial DOM utilization, a bacterioplankton inoculum at a 1:10 dilution was added to the irradiated and non-irradiated DOM fractions, and bacterial abundance and biomass production were determined (see above). Iron(II) concentrations were determined in separate irradiation experiments using the FeLume system (see above).

To test the potential effect of reactive oxygen species on bacterial DOM uptake in irradiated fractions, samples from March 1999 and April 2000 were split into three aliquots after a 4-hour irradiation. Methanol or Trolox C (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, Merck) were added to two aliquots within 60 sec after irradiation (Fig. 4.2, 4.6), and the third aliquot was left untreated as a control. Methanol exclusively scavenges hydroxyl radicals while Trolox C, a vitamin E analog, acts as a broad-band radical scavenger (Davies et al. 1988). We added methanol at final concentrations of 250 to 1000 µmol l⁻¹, depending on the DOC concentration of the sample. Trolox C was added at a much lower concentration of 500 n mol l⁻¹, corresponding to the average amounts of H2O2 formed during a 4-hour irradiation. Methanol and Trolox C were tested to act non-toxic on bacterial activity in the used concentration range. Within 60 sec of addition of methanol or Trolox C to the samples natural bacterioplankton was added at a 1:10 dilution (also to the control without scavenger) to measure bacterial abundance and biomass production (see above).

For long-term bioassay experiments DOM from April 2000 was irradiated for 10 hours (Fig. 4.2). As mentioned above, prior to irradiation HMW and hydrophobic DOM were re-diluted with MQ-UV water to reach a final DOC concentration of ~100 µmol C l⁻¹. Hydrophilic and LMW DOM were not further diluted and contained DOC concentrations ranging from 27 – 81 µmol C l⁻¹. After 10-h exposure to simulated sunlight, the samples were transferred to 500 ml erlenmeyer flasks. Nutrients (30 µmol l⁻¹...
nitrate, 300 nmol l\(^{-1}\) phosphate) were added to the HMW and hydrophobic DOM fractions to reach levels detected in 0.2 µm filtered Tagliamento river water (data not shown), followed by addition of natural bacterioplankton at a 1:10 dilution. Except for irradiation, the dark controls were treated identically. The flasks were incubated under light/dark (day/night) conditions at room temperature (22ºC) and shaken every 2 hours to prevent nutrient gradient formation. We started to determine bacterial abundance and biomass production immediately after inoculation (t\(_0\)) and kept sampling approximately every 12 hours up to a maximum of 100 hours. We also sampled for SEC at the start (t\(_0\)) and during steady-state conditions of the bioassay experiments (Fig. 4.8, 4.9). Steady-state conditions were estimated from bacterial abundance measurements.

In the field, the DOM fractions were exposed under ambient conditions to natural solar radiation in July and August 2000 (Fig. 4.2). For each DOM fraction, one aliquot was used as a dark control and was filled bubble-free in a 120 ml Winkler flask and wrapped in aluminum foil. The other aliquot was filled bubble-free in a 120 ml quartz-glass tube with a N/S glass-stopper and sealed with Teflon-tape, parafilm, and some textile-tape. The tubes and flasks were fixed on a floating mesh and incubated submersed (20 cm) in surface water of a secondary channel in the major floodplain for 6 hours (10 - 16 GMT). During incubation the surface and underwater solar radiation levels were measured every 3 hours with UV-B (290 – 320 nm), UV-A (320 – 400 nm), and PAR sensors (Macam, SD 104A-COS, SD 104B-COS) and a LI-COR 1000 data logger. Prior and after irradiation we sampled for TOC, DOC, iron, and bacterial activity measurements.

**Results and Discussion**

**Carbon and iron concentrations in the various DOM fractions**

In the Tagliamento River, the dominant form of organic carbon was dissolved organic carbon (DOC), with average concentrations ranging from 10 – 91 µmol C l\(^{-1}\) (Table 4.1). DOC as well as particulate organic carbon (POC) concentrations were low and comparable to other highly oligotrophic surface waters (Benner et al. 1997; Tockner et al. 2002). POC concentrations were calculated as the difference between TOC and DOC.
values and increased from 1 to 10% of the TOC along the main channel. These percentages are very low relative to stream size (Meybeck 1981). We observed this dominance of DOM throughout the seasons, even during post-flood conditions in August 1999. Post flooding, also high loads of inorganic carbon (carbonate, dolomite) were transported, however, 90% of organic carbon was still present as DOC. During the year, only 8 - 29% and 13 - 54% of DOM were retained by ultrafiltration and C18-solid phase extraction, respectively, comparable to what has been reported for oligotrophic marine waters (Mills and Quinn 1981; Amador et al. 1990; Benner et al. 1997). The major portion of DOM consisted of LMW compounds with hydrophilic character (data not shown).

Iron was abundant in the water column of the Tagliamento River. In the main channel, 91 - 94% of iron were present in the particulate and colloidal phase and were not passing a filter of 0.2 µm, and the remaining 6 - 9% were associated with DOM. Its distribution compares well to other freshwater systems (Emmenegger et al. 2001). In the isolated pool, located within a large island of the major floodplain, we found on average only 19% of iron in the particulate and colloidal phase. It seems that massive groundwater upwelling, infiltration by the carbonaceous substrate (gravel), and autochthonous algal production may change particle composition in that pool compared to the main channel system. Over the year, dissolved iron (DI) concentrations in 0.2 µm filtered river water samples were in the range of 3 - 57 nmol l\(^{-1}\) (data not shown). Concerning the distribution of iron in the different DOM fractions, we found that most of the DI (65 ± 4%, average ± SE, n = 21) was contained in LMW and hydrophilic DOM, sampled from the whole river corridor over the year 1999. Only in samples from April 2000 a surplus of iron was associated with HMW and hydrophobic DOM (56 ± 8%, n = 8).

**Short-term effect of DOM irradiation on bacterial DOM utilization**

Bacterial biomass production on irradiated DOM fractions was measured immediately after a 4-h exposure to simulated sunlight. Bacterial cells were incubated for 1 hour with the DOM fractions in the dark. The short-term DOM utilization of all irradiated DOM fractions decreased by up to 80% as compared to the short-term uptake of non-irradiated
DOM (Fig. 4.3). We found the same trend for all samples from the main channel and isolated pool, and also throughout the year, which may reflect a minimal spatial-temporal variability of the chemical composition of DOM in this river (unpublished data).

Fig. 4.3
Decrease (% deviation) in bacterial utilization of irradiated DOM fractions relative to bacterial utilization of non-irradiated DOM fractions at \( t_0 \). DOM originated from the main channel in the (A) headwater, (B) major, and (D) transition floodplain, and (C) from an isolated pool in the major floodplain. The exposure of the DOM fractions to simulated sunlight lasted for 4 hours. (E) shows the decrease of bacterial utilization of DOM factions irradiated for 6 hours under in-situ conditions in July and August 2000. DOM utilization
expresses bacterial biomass production (fg C cell$^{-1}$ d$^{-1}$). Data from (A) – (D) represent the annual means of 3 - 6 experiments; vertical bars indicate standard errors. Data from (E) represent the mean of 2 experiments; vertical bars indicate the mean deviation.

We also found the same level of inhibition during field studies in July and August 2000 (Fig. 4.3 E). To simulate a full solar day we exposed the DOM fractions submerged (20 cm) in surface river water for 6 hours to sunlight. Surface irradiation levels were comparable to other literature values (Herndl et al. 1993; Lindell et al. 1995), and at the maximum 5 W m$^{-2}$ for UV-B (290 – 320 nm), 82 W m$^{-2}$ for UV-A (320 – 400 nm), and 461 W m$^{-2}$ for PAR (400 – 700 nm). In the top 20 cm of the water column, the attenuation of sunlight was within 25% for UV-B, 23% for UV-A, and 10% for PAR.

Because sunlight may also directly impact bacterioplankton by damaging cell physiology and DNA (Herndl et al. 1993; Jeffrey et al. 1996), we additionally tested the direct effect of solar radiation on bacterioplankton activity. We exposed raw water samples (DOM and bacteria) as well as 0.2 µm filtered samples (only DOM) for 4 hours to simulated sunlight, and then introduced a natural bacterioplankton inoculum to the filtered non- and irradiated water samples. For both, raw and filtered, irradiated water samples we measured a strong decrease in bacterial DOM utilization (relative to non-irradiated DOM), and the inhibition was only slightly larger for irradiated raw water samples (data not shown). These additional experiments indicate that the direct photoinhibition of bacterial cells was negligible, but DOM transformation and concomitant production of reactive species were dominantly responsible for a decrease in bacterial DOM utilization.

To test the effect of reactive oxygen species (ROS) on bacterial activity, we conducted experiments with two different scavengers of ROS, methanol and Trolox C. The scavengers were added to the DOM immediately after the light source was turned off, followed by inoculation of a natural bacterioplankton community. Only for HMW DOM, methanol was effective in preventing a decrease in DOM utilization rates (Fig. 4.4 A). Based on this effect, we hypothesize that HO$^-$ or organic peroxyl radicals formed in the Fenton reaction and in subsequent thermal reactions of HO$^-$ with HMW DOM compounds were responsible for the inhibition of bacterial uptake of HMW DOM.
To test whether the Fenton reaction could play an important role in our systems, we measured ambient Fe(II) concentrations in the DOM fractions during and past irradiation. Irradiation of the DOM fractions led to the formation of Fe(II), and steady-state concentrations were reached between 1 – 5 minutes, depending on the individual DOM fraction (Fig. 4.5). Highest [Fe(II)]_{ss} were detected in irradiated HMW DOM, followed by hydrophilic, LMW and hydrophobic DOM (Fig. 4.5, Table 4.1). [Note that

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**Fig. 4.4**

Effects of methanol and Trolox C on bacterial utilization of irradiated HMW, LMW, and hydrophobic fractions sampled in April 2000 (A) directly after irradiation and (B) after 2 hours in the dark. Methanol was added at concentrations of 250 – 1000 µmol l⁻¹ and Trolox C at 500 nmol l⁻¹. DOM utilization is expressed by bacterial biomass production (fg C cell⁻¹ d⁻¹). The change in bacterial utilization of irradiated DOM fractions with and without radical scavenger additions is calculated as % deviation of utilization of non-irradiated DOM. Data points represent the mean of 2 – 3 measurements; vertical bars indicate the standard error or mean deviation.
the numbers listed in Table 4.1 are average (± SE) values from seasonally collected samples. Mean \([\text{Fe(II)}]_{\text{ss}}\) normalized to DOC concentrations, were by a factor of 4.6 higher in HMW and hydrophilic than in LMW and hydrophobic DOM (Table 4.1). Also the percentages of \([\text{Fe(II)}]_{\text{ss}}\) from dissolved iron (DI) contained in the individual fractions were generally higher in HMW and hydrophilic DOM.

![Graph A](image1)

![Graph B](image2)

**Fig.4.5**
Concentrations of Fe(II) during irradiation and after the light source was turned off in HMW and LMW DOM, and hydrophobic and hydrophilic DOM fractions. “Blank” indicates measurement of MQ-UV water at pH~9 (pH raised by adding a 25 % ammonia solution) before and after sample measurement. Dashed
lines indicate when the simulated sunlight was switched off. Fe(II) was measured by chemiluminescence detection (FeLume). We have used different calibrations curves for low and higher Fe(II) concentrations.

Regarding the kinetics of Fe(II) formation and its decay in the HMW fraction, the following observation was striking: When we turned off the light, we found that despite pH ~ 8.1, \([\text{Fe(II)}]_{ss}\) only gradually decreased and a new Fe(II) steady-state was established (Fig. 4.5 A). This phenomenon was also observed in irradiated, unfiltered water samples from a Swiss lake (Emmenegger et al. 2001). The \([\text{Fe(II)}]_{ss}\) established in the HMW fraction in the dark indicates continuous redox-cycling of iron past irradiation, Fe(III) being re-reduced by organic ligands and/or the superoxide radical (\(O_2^-\)) formed in the oxidation of Fe(II) by \(O_2\) (Voelker and Sedlak 1995; Skogerboe and Wilson 1981; Emmenegger et al. 2001). Furthermore, oxidation of Fe(II) by \(H_2O_2\), formed by iron-catalyzed \(O_2\)-dismutation, yields \(HO^+\). The pronounced effect of excess methanol on bacterial utilization of irradiated HMW DOM, suggests continuous formation of \(HO^+\) past irradiation, although at low yields at pH 8.1 (Hug and Leupin, 2003).

In the presence of Trolox C, bacterial uptake of irradiated HMW DOM was still much lower as compared to the non-irradiated sample (Fig. 4.5 A), although Trolox C is an efficient scavenger of \(HO^+\) (the second order rate constant of reaction with \(HO^+\) is \(\sim 2.2 \times 10^9\ \text{mol}^{-1}\ \text{s}^{-1}\) for Trolox C at pH 7, Davies et al. 1988, and \(\sim 7.9 \times 10^8\ \text{mol}^{-1}\ \text{s}^{-1}\) for methanol, Buxton et al. 1988). This result may be explained by the fact, that the concentration of Trolox C was too low (see Materials and methods), to scavenge the total amount of ROS including \(HO^+\). Also in irradiated hydrophobic and LMW fractions, Trolox C diminished the inhibition of bacterial production, relative to the samples without scavenger additions, but interestingly, methanol showed no effect in these fractions (Fig. 4.5 A). From this result we conclude that other ROS, possibly organic peroxides, may evolve from the photochemical transformations of hydrophobic and LMW DOM. Note that \(H_2O_2\) at concentrations of 50 - 400 mmol l\(^{-1}\) (Table 4.1) did not impact bacterial activity (data not shown).

To test the effect of DOM irradiation on bacterial DOM utilization over longer time periods, we left the irradiated fractions for 2 hours in the dark, and then added a
bacterioplankton inoculum to measure bacterial DOM utilization. In the HMW and hydrophobic fractions, the inhibition of bacterial DOM uptake had diminished after 2 hours, compared to DOM utilization immediately after irradiation, however, not in the LMW fraction (Fig. 4.5A, B). In a further experiment, we added Trolox C to one of the two irradiated HMW DOM samples, left for two hours in the dark, before measurement of bacterial DOM uptake. As Figure 4.5 B shows, Trolox C had a positive effect on bacterial DOM uptake even when added two hours after irradiation. These results suggest that ROS are formed in the dark, past irradiation, over longer time periods, however, with decreasing effect on bacterial DOM utilization. The results shown in Figure 4.5 B also indicate that changes of DOM bioavailability upon DOM photooxidation are different for different fractions (see next section).

**Long-term effect of DOM irradiation on bacterial DOM utilization**

To compare long-term bacterial growth on non- and irradiated DOM sources, we incubated bacterioplankton from the Tagliamento River in non- and irradiated HMW, LMW, hydrophobic, and hydrophilic DOM. Before inoculation with bacterial cells, the four DOM fractions were exposed to simulated sunlight for 10 h. In all bioassays, cell growth (bacterial abundance and biomass production) was monitored until and after steady-state conditions were reached (Fig. 4.6). Also in these experiments we used incubations of 1 hour with radioactively labeled leucine to measure bacterial biomass production, however, conducted these measurements at specific time intervals throughout the bioassay experiments (Fig. 4.6).

Immediately after exposure of the DOM fractions to simulated sunlight ($t_0$ in Fig. 4.6), bacterial DOM utilization was strongly inhibited in irradiated fractions. With ongoing incubation (over days), this inhibition diminished and bacteria were growing at different rates in the different fractions. Bacterial utilization of irradiated compared to non-irradiated DOM was similar for HMW DOM after 75 hours (Fig. 4.6 A), considerably higher for hydrophobic DOM after 50 hours (Fig. 4.6 C), but severely lower for LMW and hydrophilic DOM after 65 and 25 hours, respectively (Fig. 4.6 B, D). These results suggest that HMW and hydrophobic DOM, which were found to be biorecalcitrant in the Tagliamento River (data not shown), stayed unchanged or turned
Fig. 4.6
Long-time bioassay experiments on irradiated and non-irradiated DOM fractions sampled in April 2000: (A) HMW, (B) LMW, (C) hydrophobic, and (D) hydrophilic DOM. Bacterial DOM utilization is expressed by measuring bacterial biomass production (fg C cell\(^{-1}\) d\(^{-1}\)). Changes in bacterial DOM utilization are calculated as % deviation of utilization of non-irradiated DOM prior to long-term incubation.
more bioavailable upon exposure to light. On the contrary, LMW and hydrophilic DOM, which were most abundant and dominantly supported bacterial activity in Tagliamento surface waters (data not shown), turned highly biorecalcitrant upon irradiation. Interestingly, we found higher net rates of $\text{H}_2\text{O}_2$ formation (during a 4-h irradiation) in the LMW and hydrophilic fractions than in the HMW and hydrophobic fractions, suggesting that the fractions that turned biorecalcitrant upon irradiation are photochemically more reactive in terms of $\text{H}_2\text{O}_2$ formation. This trend was independent of the season, with average net rates of $\text{H}_2\text{O}_2$ formation being by a factor of 3 higher in LMW than in HMW DOM, and by a factor of 5.8 higher in hydrophilic than in hydrophobic DOM (Table 4.1).

To elucidate light-induced transformations of the different DOM fractions, we measured the molecular weight distributions of the DOM fractions prior to and after irradiation for 10 hours (Fig. 4.7). As shown in this figure, irradiation did not result in major shifts of the size spectra. However, in all fractions, irradiation caused the transformation of compounds with apparent molecular weight distribution peaking around 5-10 kD to products with lower apparent molecular weights.

We also measured the molecular weight distribution after incubation of the fractions with bacterioplankton from the Tagliamento River (Figs. 4.8 - 4.9). The size-exclusion chromatograms of irradiated and non-irradiated HMW DOM before and after incubation with bacterioplankton show that cells preferred to utilize large compounds (100 – 10 kDa) also in the irradiated HMW fraction. This contradicts literature data that report the photochemical release of bioavailable substrates with lower molecular weights from recalcitrant freshwater DOM (Wetzel et al.1995; Lindell et al. 1995). Based on organic carbon and UV detection (data of UV detection are not shown), and comparison with a suite of standard model compounds, and chemical characterization with $^{13}$C NMR (data not shown) we suggest glycosilated proteins, carbohydrates, and aliphatic/fatty acid material to appear between 100 – 20 kDa. These materials are generally bioavailable, and moreover seem to resist photolysis. The smaller compounds contained in HMW DOM (20 – 0.5 kDa) overall behave unreactive to bacterial utilization, which may be due to their aged and diagenetically altered chemical nature (unpublished data).
Fig. 4.7
Size exclusion chromatograms of non-irradiated HMW (A), LMW (B), hydrophobic (C), and hydrophilic DOM (D). Solid lines represent apparent molecular weight distributions after a 10-hour irradiation with simulated sunlight (~1 kW m\(^{-2}\)).

Non-irradiated LMW DOM dominantly supported bacterial activity and exhibited low turnover times (data not shown). Surprisingly, bacteria only consumed the larger
compounds (10 – 1 kDa) from this fraction and produced very small compounds, <0.1 kDa in size (Fig. 4.8 C). Upon irradiation, this material turned highly biorecalcitrant,

![Size exclusion chromatograms of HMW DOM (A) before and (B) after a 10-hour irradiation, and LMW DOM (C) before and (D) after a 10-hour irradiation with simulated sunlight (~1 kW m⁻²). The solid lines represent apparent molecular weight distributions after 75 hours of bacterial growth on (A) non-irradiated and (B) irradiated HMW DOM, and after 50 hours of bacterial growth on (C) non-irradiated and (D) irradiated LMW DOM. The samples were collected from the bioassay experiments after bacterial growth had reached steady-state (see Fig. 7 A, B).](image)

Fig. 4.8
reflected by unchanged size spectrum and signal intensity for the irradiated sample after 50 hours of bacterial growth (Fig. 4.7 D). The inhibition of bacterial DOM utilization may specifically be associated with the phototransformations of the 10 – 1 kDa sized compounds, because their uptake ceased after irradiation. Due to high initial bioavailability and to the very low average C:N ratio (5) of LMW DOM (unpublished data), we assume that this fraction contains an abundance of amides, aminosugars, and oligosaccharides. Photodigestion of peptides was already shown to result in a decrease in its bioavailability (Naganuma et al. 1996).

Bacterial growth on non- and irradiated hydrophobic DOM caused no change in the molecular weight distribution of both fractions, however, a strong decline in signal intensity in the irradiated fraction (Fig. 4.9 A, B). Therefore, non- and irradiated DOM after bacterial growth only differ by the extent of decrease in signal intensity. Bacterial activity measurements show that the turnover of non-irradiated hydrophobic material was low during 43 hours (Figs. 4.6 C). Due to exposure to light, this material became more bioavailable, confirming the results obtained with the bioassay experiments (Fig. 4.6 C). Chemical characterization of this hydrophobic DOM suggests the presence of terrestrial-derived and aged compounds, such as sugars, aliphatic and aromatic compounds (unpublished data), being partially photoreactive (Wetzel et al. 1995; Opsahl and Benner 1998). Therefore, we assume that phototransformations produce bioavailable compounds, which are readily consumed, however not resolvable by SEC.

Bacterial growth on non-irradiated hydrophilic DOM revealed no discernable removal of this material, but interestingly, a production of very small compounds sizing <0.1 kDa (Fig. 4.9 C). When bacteria utilized irradiated hydrophilic DOM, they also caused a release of very small compounds (Fig. 4.9 D). Like LMW material, hydrophilic DOM exhibits a low average C:N ratio (3.3), which suggests the presence of proteinaceous matter. Furthermore, the larger compounds (100 – 20 kDa) identified as glycosilated proteins in HMW DOM could neither be recovered in hydrophobic nor hydrophilic DOM. We assume that these compounds were easily hydrolyzed at low pH (required for C18-solid phase extraction), and that the smaller products contribute to the proteinaceous material expected to occur in both fractions. As mentioned above,
photodecomposition of this material may explain the strong decrease in its bioavailability (see Fig. 4.6 D).

Fig. 4.9
Size exclusion chromatograms of hydrophobic DOM (A) before and (B) after a 10-hour irradiation, and hydrophilic DOM (C) before and (D) after a 10-hour irradiation with simulated sunlight (~1 kW m\(^{-2}\)). The solid lines represent apparent molecular weight distributions after 43 hours of bacterial growth on (A) non-irradiated and after 23 hours of bacterial growth on (B) irradiated hydrophobic DOM, and after 25 hours of bacterial growth on (C) non-irradiated and (D) irradiated hydrophilic DOM. The samples were collected from the bioassay experiments after bacterial growth had reached steady-state (see Fig. 7 C, D).
Importance of sunlight and iron for riverine carbon cycling

Natural rivers like the Tagliamento River, that exhibit low TOC concentrations (<100 µmol C l⁻¹), show low light attenuation with depth, and the water column receives high doses of solar radiation over the year. The Tagliamento preserves over 60% of its aerial extension in form of non-perturbated stillwater or slow-flowing water bodies (van der Nat et al. 2002), which have an average depth of only 0.5 m (unpublished data). For this reason, in the majority of different aquatic habitats in the Tagliamento ecosystem high percentages of sunlight can penetrate to bottom levels. Practically in the whole water column, sunlight causes photosensitized reactions and photochemical transformations of DOM. In the iron-catalyzed photooxidation of DOM, reactive oxygen species are formed that may act on bacterioplankton and ‘radically’ inhibit bacterial DOM utilization. This result observed in laboratory and field experiments suggests that in sunlit surface river waters, formation of reactive species is source dependent, continuous, and critical for bacterial growth on natural DOM.

In Tagliamento sunlit surface waters, DOM undergoes severe photochemical transformations, which ultimately determine the long-term growth of bacteria on these phototransformed energy sources. The chemical reactions involved appear to be strongly dependent on the chemical composition of riverine DOM. Preceding studies have shown that HMW and hydrophobic components likely derive from leaching of soils, which contain highly altered plant material and prokaryotic biomass (unpublished data). Its origin helps to explain why this material behaves very recalcitrant to bacterial utilization. On the contrary, LMW and hydrophilic components potentially derive from the decomposition of “young”, fine POM and autochthonous microalgae biomass. This pool of molecules shows low C:N ratios (3 – 5), which may explain why they are highly bioavailable to the bacterioplankton community (see above). In the Tagliamento River, dominantly large (100 – 20 kDa) and smaller (~1kDa), possibly highly diagenetically altered compounds exhibit low photoreactivity, and therefore show no change in bioavailability upon irradiation. Some constituents of hydrophobic DOM are photoreactive and turn highly bioavailable upon irradiation. Larger components of LMW and hydrophilic DOM are also photoreactive, however, turn highly biorecalcitrant when exposed to light in surface waters.
We confirm the actual findings in the literature (Benner and Biddanda 1998; Obernosterer et al. 1999; Tranvik and Bertilsson 2001), that light can have contrasting effects on natural DOM, turning bioreactive into biorecalcitrant compounds and vice versa. Such phototransformations are highly critical for our river system, because they decrease the bioavailability of the major important energy sources for the bacterioplankton community. In consequence, this effect measured along the river continuum, suggests increasing nutritional limitation for bacteria along downstream transport, and diminishing microbial reworking of DOM. For the Tagliamento River system, we therefore propose a light-induced shift from microbial transformation processes of bioavailable substrates to hydrologic transport of photochemically altered and biorecalcitrant DOM. Others have reported that chemical, physical, and microbial reworking longitudinally decreases the nutritional value of DOM (Vannote 1981; Hedges et al. 1986; 1994). However, this is the first study that could show how light significantly impacts microbial turnover of DOM, and contributes to the transformation of organic compounds along the river continuum. For future research, it would be highly rewarding to trace light-induced structural changes of specific DOM compounds, such as bioavailable and biorecalcitrant substrates. Also the chemical characterization of organic ligands forming complexes with metals would be key for understanding the light-induced redox-cycling of metals in natural waters and their potential relationships with DOM bioavailability. Elucidating different reactive species involved would add to a more complete picture on photochemical transformations in freshwater systems.

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CHAPTER 5

Bacterial Life Strategies in a Pristine, Oligotrophic Riverine Environment: Microcolony Formation versus Living ‘Single’.

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The activity and the different life strategies of heterotrophic planktonic and attached bacteria and their phylogeny was investigated in the Tagliamento River (Italy), the last large natural river in Europe. Abundance and biomass production of planktonic and substrate-attached bacteria were inversely correlated, with low abundance but high production for attached cells and vice versa for bacterioplankton. In this oligotrophic river, low temperatures, and low total organic carbon (TOC) and soluble reactive phosphorous (SRP) concentrations limit microbial activity. Eubacteria dominated both the water column (~70%) and substrate-attached communities (~90%). Eubacteria were predominantly comprised of alpha- and beta-Proteobacteria and Cytophaga-Flavobacteria, with highest abundances of alpha-Proteobacteria in all samples. The majority of attached bacteria formed microcolonies in the oxygenated hyporheic zone. Atomic force microscopy (AFM) of bacteria, kept in a buffered aqueous phase, demonstrated that small, single, coccoid-shaped cells form small cell aggregates or colonies in association with organic and inorganic particles, microscopic algae, and organic polymeric material. AFM images indicate that attached cells develop large exopolymeric structures to randomly colonize the surface of the carbonaceous substrate. We did not detect any formation of complex bacterial biofilms. The minimal differences in community structure among bacterial samples suggest that cells, in competition for scarce resources, opportunistically explore the environment for nutrients, and therefore live either as planktonic or attached as microcolonies.
**Introduction**

Heterogeneity in the environment impacts benthic as well as planktonic microbial communities. The Tagliamento River is an extremely heterogeneous ecosystem (Fig. 5.1), grading from alpine headwater tributaries to braided floodplains, and a lowland meandering reach near the river mouth (Ward et al. 1999). However, as with other natural systems, we still have limited understanding of the interactions of microorganisms with their abiotic environment, and how they colonize substrate surfaces under turbulent or highly dynamic conditions. These interactions widely determine the distribution, activity, and community structure of microbes. Studies have shown that the diversity of different microhabitats is generally high in natural river systems, which exhibit complex channel morphology, a dynamic hydrology, and steep hydrological gradients (Ward et al. 1999; Arscott et al. 2000; Tockner et al. 2002). Benthic microenvironments are sites where organic and inorganic nutrients are likely to be trapped and stored, and may be of major importance for aquatic organisms coping with nutrient-limited conditions, such as in the oligotrophic Tagliamento River. Microenvironments may act as an ideal substrate for algal growth and foster microbial colonization. Some studies demonstrated that bacteria associated with the streambed dominated ecosystem metabolism in streams (Geesey et al. 1977; Geesey et al. 1978).

In the last two decades, research on the microbial colonization of inorganic and organic submerged surfaces in natural systems has proliferated. It has been shown that organic layers, so-called biofilms, develop and are characterized as being composed of microorganisms, exoenzymes, and organic and mineral particles embedded in abundant polymeric organic material (Golladay and Sinsabaugh 1991; Freeman and Lock 1995). Biofilm growth was documented to abundantly occur in the hyporheic and phreatic zone of rivers (Craft et al. 2000). Research on alpine streams reported that biofilms constitute the largest metabolically active bacterial biomass in low-temperature aquatic ecosystems (streams (Geesey et al 1977; Geesey et al. 1978).

Heterogeneity in the environment also impacts the benthic and planktonic microbial community structure. Fluorescent In-Situ Hybridization (FISH) can be used to identify dominant bacterial groups based on their phylogenetic identity (16S rRNA-approach), as opposed to their morphology or nutritional requirements (9Amann et al.
1990; Amann and Ludwig 2000). Studies from freshwater research suggest that the beta subclass of the *Proteobacteria* predominate in oligotrophic systems, followed by a high abundance of members of the alpha subclass (Manz et al. 1993; Kalmbach et al. 1997). Due to stochastic physical events, such as storms, *Archaea* and *Cytophaga-Flavobacteria* also may become dominant in alpine streams (Battin et al. 2001). Furthermore, there is ample evidence that the *Cytophaga-Flavobacterium* cluster may adapt to low nutrient and substrate concentrations, such as encountered in oligotropic alpine rivers. *Flavobacteria* are generally known for preferentially degrading refractory vascular plant biomass, such as cellulose and lignin, in aquatic sites (Brock et al. 2000). Geller (1986) also found that *Flavobacterium* isolates efficiently degraded refractory organic substances from lake water. O’Sullivan et al. (2002) recently reported the predominance of members of the *Cytophaga* cluster in a lotic system. However, most of these studies investigated either planktonic or benthic communities (Battin et al. 2001; Geller 1986; Pernthaler et al. 1998; Manz 1996), and studies rarely compare both communities (Crump and Baross 1996; Crump et al. 1999). Comparing dynamics in benthic and planktonic communities may be key to elucidate the coupling of benthic and pelagic processes.

On substrate surfaces, heterotrophic bacterial activity is tightly linked to the occurrence of epilithic algae, exopolymeric saccharides, and adsorbed inorganic nutrients (Battin et al. 2001, McFeters et al. 1978; Battin 2000; Espeland et al. 2001). The spatial growth structures or temporal dynamics of biofilm formation that arise from this tight coupling, however, are poorly understood. Laboratory studies with lotic bacterial communities suggest that changes in morphotypes and microcolony formation occur in early phases of biofilm development (Neu and Lawrence 1997; Manz et al. 1999). However, these studies simulate natural conditions and additionally utilize confocal laser scanning microscopy, for which sample preparation may disturb the natural structure of the analyzed material. Organisms, growing in water and attached to surfaces can, however, be pictured under in-situ conditions using atomic force microscopy (AFM). AFM was formerly used to scan the surface of dry particle or cell materials (Yalamanchili et al. 1998; Dufrene et al. 2001). Furthermore, dissolved organic matter (DOM) aggregation in water was investigated by utilizing dried DOM samples for AFM
analyses (Verdugo et al. 1999). Conversely, the application of AFM to aqueous samples, provides a non-destructive approach to study cell surfaces and cell growth in solutions.

The major aim of this study was to compare the activities and community structures of planktonic and attached bacteria, and describe their life strategies in an oligotrophic and highly dynamic alpine river. Bacterial abundance and biomass production measurements and in-situ hybridization with fluorescently labeled probes (FISH) were used to compare bacterial communities. AFM was applied to visualize cell morphologies and bacterial microcolony formation in a mesocosm experiment and also under in-situ conditions. By keeping the cells in the aqueous phase during AFM measurements, aggregate structures were kept intact and allowed us to study morphological features of bacterial growth on natural substrate surfaces.

Figure 4.1
Catchment site of the Tagliamento River with important sampling stations marked by large grey patches: Main channel in headwater floodplain (R2MC), in major floodplain (R4MC), and in transition floodplain.
(R5MC), and isolated pool in major floodplain (R4P). Find the most important tributary streams, the Arzino, But, Degano, and Fella in the catchment area. The little inset shows that the river is located northeast Italy, Europe.

Material and Methods

Study site and sample collection

Bacterial samples used in this study originated from surface waters collected from the river corridor of the Tagliamento River, northeast Italy. The river is classified as the last large natural river in Europe (Mueller 1995), flowing unrestrained from the alpine headwater reaches to its mouth in the northern Adriatic Sea, and forming a complex channel morphology, characterized by constrained, braided, and meandering gradients (Ward et al 1999; Arscott et al. 2000). Samples collected were part of a larger study in which water was collected seasonally from March 1999 to July 2000 from the main channels at three downstream stations (R2MC, R4MC, and R5MC) as well as from one isolated pool in R4 (R4P) (Kaiser et al. in submission) (Fig. 5.1). Water levels during sampling varied from low- to high-flow conditions (Arscott et al. 2000). The in-situ incubation experiments took place during medium-flow conditions in R4MC in July 2000. Throughout the year and during analyses, the main channel river water was characterized by temperatures of 10.9 ± 2.5°C, pH’s of 8.13 ± 0.14, and alkalinities of 2.8 ± 0.5 mM. Bacterioplankton samples were collected with clean 50 l high-density polyethylene (HDPE) carboys and transported back to the field station, located in R4. Carbonaceous substrate, such as large (5 – 20 cm) limestone pebble and gravel, were collected in July 2000 using a clean container for future mesocosm experiments (see below). Immediately following collection, the water samples and the substrate material were stored at 4°C in the dark. Within 2 days of collection all the materials were brought back to the laboratory (EAWAG, Switzerland).

Bacterial surface colonization was studied under in-situ conditions in July 2000. Calcareous gravel was taken at the same location where water samples were collected. Before drying, the gravels were marked with red-colored elastics, and freshly cleaved 1 cm² mica-plates (muscovite, KAl₂[AlSi₃]O₁₀(OH)₂) were fixed onto the surfaces. Then
the gravel was immediately introduced back into the river at the same site. Bacterial activity, total organic carbon (TOC), and nutrient (nitrate, ammonium, soluble reactive phosphorus) concentrations were measured from the water samples collected for the mesocosm experiment (control raw river water). After a 4-day inoculation, the exposed gravels were collected from the river bed. The mica-plates were removed, carefully placed into petri-dishes, and covered with 5%-glutaraldehyde solution (Merck) to stabilize bacterial cells and inhibit further cell growth. The petri-dishes were sealed and stored at 4°C in the dark for further AFM analysis. Within 2 days of collection the harvested bacteria on the mica-plates were brought back to the laboratory (EAWAG, Switzerland).

**Mesosom experiments**

A clean 40 l reactor tank (HDPE) was used to set up the mesocosm experiment. The reactor was placed in a culture room with constant temperature of 15 °C and illuminated in a 12 h light/12 h dark (day/night) cycle. The bottom of the reactor was covered with carbonaceous substrate (pebble and gravel), collected from the Tagliamento River, to simulate the oxygenated surface region of the hyporheic zone. Clean tygon-tubes with air-stones fixed at one end were introduced to the system. The tubes and air-stones were mounted to the carbonaceous substrate. Then 30 l of raw river water was introduced into the reactor as the main source of bacterial cells, organic carbon and nutrients. Clean (0.2 µm filtered) air was supplied at a constant rate to the mesocosm. Strong air-flow (200 l min⁻¹) was applied to simulate strong mixing in and on the surface region of the mainstem sediments. We took effort to imitate a 0.5 m deep water column, typical for the sampling site and characterized by turbulent mixing due to a dynamic hydrological regime and a water-flow of ~1m s⁻¹. The tank was covered with pyrex-glass plates to prevent carbon or nutrient contamination. Bacterioplankton abundances, pH, TOC, and nutrient (nitrate, ammonium, soluble reactive phosphorus) concentrations were monitored over time as the bacterial culture was developed for 7 days. Every second day, one half of the water volume was replaced with raw river water stored in 50 l carboys at 4°C in the dark. After each volume replacement samples for DOC and nutrient analysis were taken. Then, after one week of culturing, mica-plates were fixed onto the surfaces of the
carbonaceous substrate. Then the gravel was again introduced into the mesocosm. The culture was further developed for ~4 weeks to induce microbial colonization of the substrate surfaces. Water was replenished every second day, and cell numbers as well as the chemical composition were monitored as mentioned before.

After ~4 weeks the culture was harvested. Bacteria were collected from the planktonic phase and the substrate surface for bacterial activity measurements, AFM and FISH characterization. Bacterioplankton activity (abundance and biomass production) was determined from ambient samples taken from the mesocosm, whereas for AFM and FISH, the cells were concentrated by centrifugation. Bacterioplankton from three 50 ml samples were each concentrated to a final volume of 2 ml. One sample was used for AFM and the other two replicates for FISH. Samples for AFM were stabilized with 5%-glutaraldehyde solution. Bacteria from the substrate surface were removed and collected with a 10 ml pipette. The end of the pipette tip was cut to enlarge the opening (~1 cm) helping to better scratch/suck bacteria from the substrate. Samples containing attached bacteria were treated like bacterioplankton samples, and also used for bacterial activity, AFM, and FISH measurements. For cross-comparison with bacteria used for inoculation and culturing, cells were also collected from raw river water (control) stored in 50 l carboys and processed like samples from the mesocosm. To visualize intact bacterial growth on the substrate surface, gravels with mica were removed from the mesocosm and the mica-plates harvested. The plates were carefully placed into petri-dishes and covered with 5%-glutaraldehyde solution to stabilize bacterial cells and inhibit further cell growth, after which samples were immediately analyzed by AFM.

**Bacterial abundance and biomass production**

Samples (10 ml) for microscopy were preserved with a filtered borax-buffered formaldehyde solution (5% final concentration) and stored refrigerated in the dark. Bacterial abundance was determined within two weeks of sample collection by epifluorescence microscopy (Olympus BX50, 1,000 magnification) of DAPI (4’,6-diamidino-2-phenylindole)-stained cells (Porter and Feig 1980).

Bacterial production was estimated from protein synthesis measured in triplicates with two formalin-killed blanks (2% final concentration). [³H]leucine was added to a 20
nM final concentration to each of the 5 ml subsamples and incubated in the dark at 20°C for 60 minutes (Kirchman et al. 1986). After incubation, the samples were filtered onto nitrocellulose filters (GSWP, 0.2 µm pore-size, 25 mm diameter, Millipore) and rinsed twice with 5 ml of chilled 5% trichloroacetic acid. The filters were placed in scintillation vials with 10 ml of scintillation cocktail (Insta-Gel, Packard) added. Radioactivity was assessed with a liquid scintillation counter (Packard Tri-Carb 2000) by external standard ratio technique. A conversion factor of 3.1 µg C nmol\(^{-1}\) leucine was used to convert leucine incorporation into the bacterial biomass production (Simon and Azam 1989).

**Bacterial community structure analyses by Fluorescent In-Situ Hybridization (FISH).**

The following oligonucleotide probes (Microsynth, Balgach) were used to describe the microbial communities: EUB338, EUB338II, and EUB338III (16S rRNA, positions 338 to 355) for members of the domain *Bacteria*, ALF1b (a6S rRNA, positions 19 to 35) for the alpha subclass of *Proteobacteria*, BET42a (23S rRNA, positions 1027 to 1043) for the beta subclass of *Proteobacteria*, and CF319a (16S rRNA, positions 319 to 336) for the *Cytophaga-Flavobacterium* cluster (9, 16, 18, 34, 35). Probes were labeled with the indocarbocyanine fluorescence dye CY3 (Microsynth). To ensure optimal stringency conditions, the unlabeled probe GAM42a served as competitor for BET42a (Manz et al. 1993; Manz et al. 1999).

For storage until FISH, recovered cells were resuspended in 5 ml sterile phosphate-buffered saline (PBS) MQ-UV water (Millipore) at pH 7.4, and containing 8 g NaCl, 0.2 g KCl, 1.44 g Na\(_2\)HPO\(_4\), and 0.24 g KH\(_2\)PO\(_4\) per liter MQ-UV water. Then a 4% paraformaldehyde solution was added and the samples were fixed for 1 hour at room temperature. Afterwards, the cells were washed twice with PBS. The fixed cells from each sample were suspended in 2 - 3 ml of a solution of 50% PBS and 50% ethanol (vol/vol). The cell suspensions were stored at −20°C for FISH. The hybridization procedure of Amann et al. (1990) was used. Glass slides were coated with gelatin. 10 µl of sample was applied on a well of the coated slide, dried for 3 hours at 46°C, and subsequently dehydrated in solutions of 50%, 80% and 100% ethanol in PBS (vol/vol) for 3 min each. To start hybridization, 9 µl of hybridization buffer and 1 µl of a fluorescently
labeled probes (50 ng oligonucleotides µl⁻¹) were added to each well. The hybridization buffer consisted of 0.9 M NaCl, 20 mM Tris-HCl (pH 8), 0.01% SDS, and the appropriate amount of formamide (35% or 20% for ALF1b, and 0% for EUB-mix). The hybridization was conducted at 46°C in a humidified chamber for 90 minutes. Following hybridization, the glass-slides were washed for 10 minutes at 48°C with phosphate buffer (pH 8) containing NaCl, 20 mM Tris-HCl (pH 8), 5mM EDTA (no EDTA with 0% formamide) and 0.01% SDS. Then the samples were counterstained with DAPI (10 µg ml⁻¹) for 5 minutes at room temperature, and mounted in Citifluor medium (Citifluor) for epifluorescence microscopy. Microscopy was performed on an Olympus BX50 microscope, equipped with the filters HQ-CY3 and HQ-DAPI (Analysentechnik). Digital images were taken with a CCD camera (type Sensy, Photometrics). The software METAVIEW (Visitron) was used to acquire bacterial images and quantify labeled cells.

**In-situ Atomic Force Microscopy (AFM) to analyze bacterial images**

Concentrated and fixed bacterial samples as well as bacteria grown on mica-plates were directly used for AFM analyses. Conventional in-situ analysis were performed with an O-ring between the specimen and the tip holder (Digital Instrument Manual). In order to avoid frictions, due to the O-ring, we performed the analyses on a Teflon® plate, previously taped on the specimen holder. Thus, based on its hydrophobicity, the Teflon plate allowed us to observe the specimen in water without ruining the piezoelectric scanner. The mica-plate once taped on the Teflon plate could be directly pictured in the water. A drop of the cell suspension was disposed onto the mica and air dried. The drying process enables us to avoid tip contamination with cells. Mica-plates with attached cells recovered from the mesocosm or Tagliamento River were directly analyzed without an air drying process. In order to balance forces between tip and specimen, a phosphate buffer (pH 7) was used during the analysis.

The AFM measurements were performed in contact mode with a Nanoscope III controller, Nanoscope III v3.2 software (Digital Instrument), and standard SiN₄ cantilevers (for contact mode). For all experiments, we used V-shaped cantilever (length ~100 µm) and tips with a square pyramidal shape (half angle of ~35°). Images were recorded with a 15 µm scanner (E-type, Digital Instrument). The Set-point was set
between 1.5 - 2 volts with a scan speed of 1 - 1.5 Hz, and the integral gain and proportional gain between 4 and 5. These settings were applied to all the recorded images, and a real-time plane fit filter was applied to each scanned image.

**TOC and nutrient analysis**

Water samples (20 ml) were collected directly after sampling in acid-rinsed, muffled 100 ml Schott-glass bottles (Duran), sealed with clean caps, and stored frozen until analysis. After acidification to pH~3 (2 M HCL) and 5-min sparging, TOC concentrations were determined by high temperature catalytic oxidation with a Shimadzu 5050A analyzer (Benner and Strom 1993; Benner et al. 1997). Concentrations of ammonium (NH$_4^+$), nitrite plus nitrate (NO$_2^-$ + NO$_3^-$), and soluble reactive phosphorous (SRP) were analyzed as reported in Arscott et al. (2). Dissolved inorganic nitrogen (DN) was calculated as [NO$_2^-$] + [NO$_3^-$] + [NH$_4^+$].

**Results**

**TOC and nutrient concentrations, and bacterioplankton activity in the Tagliamento River**

TOC concentrations ranged from 35 to 102 µM C, with an average (± SE) value of 66 ± 5 µM C in surface waters of the Tagliamento mainstem during the course of the study. During the field study in July 2000, TOC was 91 µM C in mainstem waters. In contrast to DIN which was much higher with an average (± SE) concentration of 50 ± 11 µM N (Arscott et al. 2000), and 43 µM N in July 2000. Soluble reactive phosphorous (SRP) concentrations were low, with an average (± SE) value of 0.12 ± 0.01 µM P (2), and 0.1 µM P in July 2000.

Over the year and along the river, bacterial abundances in raw river water were generally low with the highest average (±SE) found in R4MC (3.92 × 10$^8$ ± 0.87 cells l$^{-1}$) and lowest average measured in R2MC (1.37 × 10$^8$ ± 0.46 cells l$^{-1}$) (Fig. 5.2 A). Over the year, bacterial biomass production was also low but, interestingly, revealed a reverse trend with highest average (± SE) value in R2MC (53 ± 25 fg C cell$^{-1}$ d$^{-1}$) and the lowest average (± SE) value in R4MC (17 ± 6 fg C cell$^{-1}$ d$^{-1}$) (Fig. 5.2 B).
Bacterial abundance and biomass production measured from unfiltered surface river water sampled from the main channel in the headwater (R2), major (R4), and transition (R5) floodplain, and from one isolated pool in R4. Data represent annual means (n = 6 - 7); vertical bars indicate standard errors.

TOC and nutrient concentrations, and bacterioplankton activity in the mesocosm experiment

The TOC, DIN, and SRP concentrations did not fluctuate over the time of the experiment. TOC stayed at 90 ± 7 µM C, DIN at 43 ± 5 µM N, and SRP at 0.11 ± 0.03 µM P. During ~4 weeks, the culture was characterized by pH 8.2 ± 0.1 and 15 ± 1°C.

Bacterioplankton were introduced to initiate mesocosms using raw river water. Raw river water (control) used for culturing contained ~3.9 × 10^8 cells l^-1, whereas 3.5 × 10^8 cells l^-1 were counted from mesocosm water samples at the start of the experiment. In the first two weeks of the culturing, planktonic cells slowly increased in number from 3.5 to 18.1 × 10^8 cells l^-1. Abundance remained relatively constant until the termination of the experiment, showing a final amount of 18.7 × 10^8 cells l^-1 after ~4 weeks of culturing (Fig. 5.3 A). DAPI-stained bacterioplankton cells from the culture were dominated by
small cocci (data not shown). Abundance of bacteria living on the substrate surface, collected at the end of the experiment (after ∼4 weeks) was $0.54 \times 10^8$ cells l$^{-1}$ (Fig. 5.3 A).

Biomass production of bacterioplankton from control raw river water, and bacterioplankton and substrate-attached cells from the mesocosm were measured at the end of the experiment. Bacterioplankton from control raw river water produced $52 \pm 5$ fg C cell$^{-1}$ d$^{-1}$ and bacterioplankton from the mesocosm $8.7 \pm 1$ fg C cell$^{-1}$ d$^{-1}$, whereas attached cells from the mesocosm were much more productive with a rate of $249 \pm 10$ fg C cell$^{-1}$ d$^{-1}$ (Fig. 3 B).

![Fig. 5.3](image.png)

**Fig. 5.3**
Bacterial abundance and biomass production measured from bacterioplankton from raw river water (control), and bacterioplankton and substrate-attached bacteria from the mesocosm experiment. All samples were collected at the end-point of the mesocosm experiment (∼4 weeks). The control river water was used for mesocosm cultivation. Data represent means (n = 3); vertical bars indicate standard errors.
Bacterial communities structures analyzed by Fluorescent In-Situ Hybridization (FISH)

In the mesocosm experiment, we found that eubacteria dominated the planktonic and attached bacterial communities. They constituted ~70% of the water column and ~90% of the substrate-attached population (Table 5.1). By using specific probes we identified ~67% of the eubacteria in the control raw river water used for feeding the mesocosm, ~38% in the planktonic phase of the mesocosm, and ~88% attached onto the mesocosm substrate surface (Table 5.1). Bacterioplankton in control raw river water and in the mesocosm showed a similar proportion of alpha-Proteobacteria (~25 and ~29%, respectively), however, contained a lower abundance of the beta-subclass of the Proteobacteria and of Cytophaga-Flavobacteria (Table 5.1). Substrate-attached bacteria in the mesocosms showed the highest abundance of all bacterial groups tested, with highest percentages for alpha-Proteobacteria (~41%), followed by beta-Proteobacteria (~30%), and Cytophaga-Flavobacteria (~17%). Overall, it appeared as if the alpha-Proteobacteria dominated the microbial community in the mesocosm system.

Table 5.1

Community structure of bacteria sampled from control river water, and from the planktonic phase and substrate surface of the mesocosm. Control river water was used for mesocosm cultivation. The community structure was analyzed by FISH.

<table>
<thead>
<tr>
<th>Bacterial sample</th>
<th>% Eubacteria of total DAPI count</th>
<th>% alpha-Proteobacteria</th>
<th>% beta-Proteobacteria</th>
<th>% Cytophaga-Flavobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control river water</td>
<td>72 ± 6</td>
<td>29 ± 6</td>
<td>19 ± 12</td>
<td>19 ± 12</td>
</tr>
<tr>
<td>planktonic</td>
<td>68 ± 10</td>
<td>25 ± 14</td>
<td>7 ± 3</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>benthic</td>
<td>92 ± 12</td>
<td>41 ± 4</td>
<td>30 ± 13</td>
<td>17 ± 6</td>
</tr>
</tbody>
</table>
**Bacterial image analyses by in-situ Atomic Force Microscopy (AFM)**

Small cocci comprised the largest biomass of the bacterioplankton communities, as determined by DAPI-stain and epifluorescence counting. In addition, AFM was successfully used to image the morphology of planktonic and substrate-attached bacteria. The cells exhibited an average (± SE) size of 200 ± 30 nm, and average (± SE) cell length:width ratio of 1.23 ± 0.31. Morphotype analyses of bacterioplankton from control raw river water and the planktonic phase of the mesocosm confirmed that small coccid-shaped cells dominated the water column in the mesocosm (Fig. 5.4 A, B). In addition, some rod-shaped cells could be found in the planktonic communities (Fig. 4 A, B). Note, that bacteria were of different sizes in Fig. 4 A, B, due to attachment of cells to each other, and their appearance as one larger cell. Small bacteria also dominated the substrate-attached community (Fig. 5.4 C) with an average (± SE) cell length:width ratio of 1.27 ± 0.57 for all samples proved in the whole mesocosm.

AFM also was successfully used to characterize the growth of attached bacteria on carbonaceous pebble and gravel. Bacterial colonies growing on mica-plates, which were implanted onto substrate surfaces, could be transferred to the microscope in the aqueous phase avoiding dramatic change in physical or chemical conditions. This property makes AFM a desirable and fast tool for characterization of bacterial growth structures. Using AFM we characterized the long-term growth of attached bacteria under laboratory (mesocosm) and in-situ conditions. We did not detect the formation of an intense biofilm on mica-surfaces, whether surfaces were incubated in the mesocosm or in the river-bed. We observed the formation of microcolonies and patchy cell-aggregates associated with long extracellular polymeric structures, growing radially away from the cells (Fig. 5.5 A, B; Fig. 5.6). Abundant polymeric structures were found in microcolonies formed under natural conditions in the Tagliamento River (Fig. 5.6).
FIG. 5.4
AFM of the bacterial cell morphologies: (A) bacterioplankton from control raw river water (height image represented in 3D), (B) bacterioplankton from the mesocosm (height image represented in 3D) and (C) substrate-attached cells from the mesocosm (deflection image).

Fig. 5.5
AFM images of bacteria grown on mica-plates in the mesocosm experiment. (A) – (C) represent true replicates harvested after ~4 weeks: (A) and (B) height image, 3D, and (C) height image.
AFM images of bacteria growing under in-situ conditions on a mica surface. Cells from both samples were grown for 4 days under in-situ conditions in the mainstem of the Tagliamento River. (A) height image and (B) deflection image.

**Discussion**

**Downstream pattern of bacterial activity**

Low TOC, SRP, and contrasting high DIN concentrations in surface waters of the Tagliamento River suggest strong carbon- and phosphorus limitation for bacteria (Arscott et al. 2000; Kaiser et al. in submission). Bacteria are regarded to make up a significant portion of the living biomass in freshwater systems and are major consumer of dissolved organic compounds (Cole et al. 1988). Therefore, the availability of the above mentioned energy and nutrient resources is crucial for their activity in this lotic ecosystem. An extensive characterization of DOM isolated from the Tagliamento River water revealed that up to 40% of the bulk DOC is aged and diagenetically altered, rendering it strongly biorecalcitrant to bacterial utilization (Kaiser et al. in submission; Kaiser et al. 2003). Thus, the DOM pool transported in this oligotrophic alpine river appears to strongly control bacterial growth. Small cell sizes with coccoid shape, indicative of starvation in
oligotrophic surface waters (Battin et al. 2001), are inferred from enumeration of DAPI-stained cells and suggest resource limitation. However, due to low average water temperatures, we also believe that not only resources but also temperature may control bacterial activity (Nedwell 1999). Consequently, bacterial activity may be controlled by multiple factors in the Tagliamento River.

Bimonthly measurements of bacterioplankton activity reflected these severe limiting conditions. Results demonstrate that bacterial activity (on a per cell basis) is not linked to the size of bacterial populations but differing DOM source and composition (Fig. 5.2). Bacterial numbers were generally highest in the major floodplain and lowest in the headwaters, whereas bacterial production was always lowest in the major floodplain and highest in the headwaters. Regarding bimonthly measurements, bacterial abundance and production show low seasonal variability within reaches (Fig. 5.2). This may be supported by the finding that the chemical composition of DOM, the major energy source of bacteria, does not change spatially or temporally in the Tagliamento River (Kaiser et al. in submission).

However, the bioavailability of organic materials may vary along the river continuum, being overall higher in the headwaters. Several studies on the Tagliamento ecosystem have documented, that due to habitat complexity, the river system receives and retains high loads of organic biomass (Gurnell et al. 2000; Nat van der 2002). Fine suspended organic particles (>0.7 µm) are highly reactive and remineralized fast in a downstream direction (Kaiser et al. in submission). These particles show very high bioreactivity and fast turnover in the headwaters. Their decomposition releases bioavailable dissolved organic substrates and inorganic nutrients, explaining elevated bacterial biomass production in that stretch of the river. Interestingly, this contribution does not cause an increase in bacterial abundance. Bacterial numbers may possibly be controlled by high fungal biomass, strongly associated with the break-down of particulate organic matter in streams (Nat van der 2002). On the contrary, massive lateral transport of organic materials was shown to be highest in the major floodplain (Nat van der 2002; Kaiser et al. in submission). From these materials, large woody debris and soil-derived organic matter constitute the largest standing stock of organic biomass in the river ecosystem. However, due to large sizes and a refractory nature (Kaiser et al. in submission) they are not
directly accessible to aquatic microorganisms. Additionally, bulk DOM may contain up to 40% of biorecalcitrant constituents, which appear to comprise a fairly constant fraction of DOM during longitudinal transport (Kaiser et al. in submission). Therefore, it appears that in the major floodplain either resource limitation of dissolved bioavailable organic and inorganic substrates leads higher cell abundances (Morita 1982) or slightly elevated SRP concentrations (Arscott et al. 2000) may, e.g., favor cell division relative to biomass production (Egli 2000). Furthermore, competition with fungi or grazing pressure may be diminished in the highly heterogeneous major floodplain.

Studies on marine and freshwater regimes report the dominance of small and planktonic cocci in oligotrophic waters (Battin et al. 2001; Fuhrmann et al. 1989). Their higher surface:volume ratio allows them to compete more readily for limiting resources. In contrast to the oceanic regime or to alpine streams devoid of vegetation and input of plant biomass, the Tagliamento River offers a heterogeneous environment and close coupling of terrestrial and aquatic sites. Furthermore, in the Tagliamento River, a low average depth of ~0.5 m for different water bodies suggests intense coupling of pelagic and benthic processes. It seems that this high habitat diversity, organic matter influx and retention in the major floodplain, lead to the development of intense biofilms on substrate surfaces of the hyporheic zone at the expense of the small unproductive bacterioplankton. The mesocosm experiment enabled us to better compare bacterial activities of planktonic bacteria from the water column and of substrate-attached bacteria. Higher abundances of planktonic cells exhibiting low productivity and very low abundances of substrate-attached bacteria showing enormously high biomass production were actually measured in the mesocosm (Fig. 5.3). Other studies suggest that epilithic microalgae, non-living organic substrates, and embedded inorganic nutrients form a rich organic layer on carbonaceous pebble and gravel (Battin 2000; Battin et al. 2001). Attached bacteria, living in close coupling with this rich matrix, may effectively use resources entrapped or adsorbed. Hence, in this lotic system (especially in R4MC), although planktonic cells dominate and have to cope with a multiple limitation (as discussed above), a small number of bacteria colonizes the carbonaceous substrate surface, and thereby experiences no resource-limitation and is highly productive as a result.
Bacterial communities structures

Although the Tagliamento represents a highly complex river system and bacterial communities living in the pelagic and benthic environment exhibit contrasting activities, they are phylogenetically similar. We explain this similarity in community compositions mainly by the severe nutritional limitation and invariable low temperatures (see above). We found that the majority of planktonic and attached cells fell into the alpha subclass of the Proteobacteria (Table 5.1). This result is not consistent with other reports from oligotrophic freshwater systems ranging from alpine lakes (Pernthaler et al. 1997; Alfreider et al. 1996) to streams (Battin et al. 2001). Our data contradict the recent findings that beta-Proteobacteria occasionally account a large proportion of the Eubacteria in planktonic (Gloeckner et al. 1999) or lotic communities (Battin et al. 2001; Manz et al. 1999). In the Tagliamento River, Beta-Proteobacteria were consistently lower in abundance, particularly in the planktonic community (Table 5.1).

We further detected members of the Cytophaga-Flavobacterium cluster in the planktonic and attached communities, with highest abundance in the benthic environment (Table 5.1). Several studies showed that members of the Flavobacterium cluster are adapted to low temperatures and low organic carbon and inorganic nutrient concentrations, and bacterial isolates effectively utilize biorecalcitrant organic substrates compared to other strains (Geller 1986; Noble et al. 1990). Due to their tolerances for these physical and chemical constraints, we would expect higher abundances of Flavobacteria in the Tagliamento river system. They may be out-competed by strains that more effectively utilize the slightly larger proportion of bioavailable compounds within the dilute pool of DOM (Kaiser et al. in submission). Further, we explain their relatively low abundances by the very low concentrations of their primary nutritional sources, such as refractory and vascular plant biomass derivatives, within bulk DOM (Kaiser et al. in submission). Elevated numbers of Flavobacteria in the benthic community may be explained by a higher presence (retention) of refractory DOM in benthic microhabitats.

Life strategies in an oligotrophic alpine river system

As inferred from epifluorescence microscopy (DAPI-counting) and AFM, bacterioplankton was uniformly comprised of small cocci. Other work (Moriarity and
Bell 1993) has implied that cell size is an indicator for the trophic status of a bacterium. Small sizes normally represent starvation, which in this river is caused by low temperatures and low supply of bioavailable substrates. As found from bimonthly bacterial abundance measurements, cocci dominated the downstream portions of the river and also throughout the year. We have already explained this finding by the spatially and temporally invariable chemical composition of DOM (see above).

In the mesocosm as well as in the river-bed, small cocci were found to colonize carbonaceous pebble and gravel. It is of interest, that in both systems we did not observe the formation of vast biofilms, but rather aggregates of cells forming microcolonies (Fig. 5.5 C). Other studies have reported microcolony formation in the early phases of lotic biofilms (Neu and Lawrence 1997; Manz et al. 1999). Thus, it appears that in the temperature- and resource-limited Tagliamento ecosystem only low levels of biofilm complexity are reached. AFM analyses also enabled us to detect the presence of large substrate-attached exopolymeric structures under laboratory (mesocosm) (Fig. 5.5 A, B) as well as in-situ conditions (Fig. 5.6). These structures developed radially away from cell aggregates. We assume cell aggregates because their size points to the presence of several small cocci or rods that are attached to each other. These exopolymers may fulfill multiple functions, such as transient carbon and nutrient storage enabling induced enzymatic processing, or surface-activated growth (Battin et al. 2001). If bacterial growth depends on biorecalcitrant organic substances, cells try to immobilize these substances in an organic matrix for further enzymatic digestion (Battin et al. 1999). In cold waters bacteria also require higher substrate concentrations because of reduced substrate affinity in membrane transport proteins (Nedwell 1999; Wiebe et al. 1992). In cold Tagliamento River water DOM concentrations are low, and up to 40% of this pool possesses a refractory chemical nature and low bioavailability (Kaiser et al. in submission). It may be advantageous for bacteria to trap organic substrates needed for growth in extensive polymeric structures (Battin et al. 2001), especially biorecalcitrant compounds, which need further extensive enzymatic processing (Chrost 1991). There is ample evidence that heterotrophic bacterial enzymatic activity is tightly linked to the occurrence of a rich organic biofilm, and the production and release of bacterial ectoenzymes is tightly bound to substrate availability in the biofilm (Espeland et al. 2001; Chrost 1991). Although only
low levels of biofilm complexity are reached in the Tagliamento River, large exopolymers are developed. We hypothesize that their formation and abundance is primarily determined by the availability of nutrients. In addition, these polymeric structures may enhance physical attachment. It had been shown, that starved bacteria activate the production of similar structures to adhere to surfaces (Kjelleberg and Hermannsson 1984), and further to concentrate energy resources and nutrients.

To cope with complex environmental conditions in highly dynamic lotic systems, bacteria have to develop multiple adaptations or strategies to survive. We were able to demonstrate that the majority of bacteria, which are small and coccoid-shaped, live under severe temperature- and resource-limitation in the pelagic regime. Only a small proportion of cells grows adhered to pebble and gravel in the hyporheic zone. However, these cells were found to be highly productive and to form microcolonies and large exopolymers within a rich organic matrix. The use of these exopolimeric structures are still ambiguous, however they proved advantageous for the survival and growth of microbes in cold and oligotrophic rivers. Because of minor differences in planktonic and benthic community compositions, we further propose that bacteria in the Tagliamento River are in competition for scarce resources and opportunistically explore their microenvironments possibly switching between planktonic or substrate-attached states.

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References


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Conclusions and outlook

From my Ph.D. research I conclude that the high variability of natural rivers in space and time (Petts and Amoros 1996; Ward 1998) results in an amazing complexity of sources, reactivities, transformation pathways, and fates of riverine organic matter. Due to tight coupling of terrestrial and aquatic systems, terrestrial materials (such as woody debris and soil-derived organic matter) dominate the standing stock of organic matter within complex floodplain habitats and contribute to the pool of organic compounds transported by Tagliamento surface waters. In this river, dissolved organic matter (DOM) is another important pool of organic matter and is partly derived from plant matter and soil biomass. A small proportion of these organic moieties (20 – 40%) is of high-molecular weight, with partly hydrophobic character, strongly aged, and shows a high degree of diagenetic reworking. These chemical features cause low bioreactivity of these DOM compounds. Consequently, its transport by surface flow appears to be dominantly conservative.

My Ph.D. studies show that what drives the river energy and nutrient flow is a diagenetically young and fast cycling pool of low-molecular weight and hydrophilic substances. They seem to be of low age and derive from the decomposition of suspended organic particles and autochthonous microbial production. Their reactive and bioavailable nature explains their fast downstream processing and the uniform chemical signature of bulk DOM sampled along the river continuum. This DOM component constitutes 60 – 70% of bulk riverine DOM and mainly supports bacterial activity in Tagliamento surface waters. Although bacterial uptake of bioavailable compounds is fast (calculations revealed that potentially half of the pool of bioavailable compound can be consumed by bacterioplankton during transport from the headwaters to the river estuary), concentrations of bioavailable DOM are low and contribute to a multiple limitation, composed of low temperatures and low dissolved inorganic phosphorus concentrations that suppresses bacterioplankton activity. In contrast to bacterioplankton, surface-attached growth was found to foster bacterial activity, and attached cells were highly productive and formed microcolonies and large exopolymers within a rich organic matrix. Interestingly, attached growth resulted in only low levels of biofilm complexity.
Also the use of these exopolymers is still ambiguous, although they proved advantageous for the survival and growth of microbes in cold and oligotrophic rivers. Because of minor differences in planktonic and benthic communities compositions, I further propose that in the Tagliamento River bacteria in competition for scarce resources opportunistically explore their microenvironments and may switch between planktonic or substrate-attached states. I believe that future research on the spatial structure and temporal formation of these natural bacterial assemblages, would help to better understand the life strategies of the most important living biomass, responsible for the destruction and turnover of organic materials, in a dynamic and oligotrophic riverine environment. It would further add to explain riverine fluxes and cycling of the most important elements for life.

A major part of my Ph.D studies was the investigation of photochemical DOM transformations as well as bacterial DOM uptake, which should add to better understand the extent of riverine processing of organic materials. Sunlight was found to strongly impact riverine carbon cycling, and thereby to affect bacterial DOM utilization in two ways: (i) short-term inhibition by reactive oxygen species formed in the iron-catalyzed photooxidation of DOM compounds and (ii) long-term inhibition resulting from strong photoalteration of DOM compounds, rendering them biorecalcitrant to bacterial uptake. Phototransformations are highly critical for the Tagliamento River, because they decrease the bioavailability of the major important energy sources for the bacterioplankton community. This effect, in consequence, suggests increasing nutritional limitation for bacteria along downstream transport, and diminishes the microbial reworking of DOM. For this river system, we therefore propose a light-induced decrease of microbial transformation processes of bioavailable substrates during downstream transport and the production and transport of photochemically altered and biorecalcitrant DOM. This is the first study that could show how light significantly impacts microbial turnover of DOM and contributes to the transformation of organic compounds along the river continuum. For future research, it would be highly rewarding to trace light-induced structural and molecular changes of specific DOM compounds, such as bioavailable and biorecalcitrant substrates. Also the chemical characterization of organic ligands forming complexes with metals would be key for understanding the light-induced redox-cycling of metals in
natural waters and their potential relationships to DOM bioavailability. Elucidating different reactive species involved would add to a more complete picture on photochemical transformations in freshwater systems.

From this research, we propose that small and highly diagenetically altered moieties are dominantly exported to the northern Adriatic Sea, and contribute to the large pool of aged, highly degraded, and marine LMW DOM. It has been shown that changes in salinity may induce precipitation (floculation) of DOM in the river plume (Sholkovitz 1976). However, this is only reported for the larger DOM constituents. We do not know what happens to LMW and hydrophilic substances when they enter the marine environment. It has been shown that terrigenous organic matter contained in HMW DOM only resides for up to a maximum of 130 year in the ocean (Opsahl and Benner 1997). But, we are not able to investigate the residence time of terrigenous LMW DOM in marine waters, because a high abundance of salts prevents isolation and further chemical characterization. Highly degraded terrestrial-derived DOM of lower molecular weight may survive in marine waters for long time-scales and contribute to the pool of highly biorecalcitrant and old marine DOM (Williams and Drueffel 1987). Further, comparisons of DOM processing in natural and manipulated river systems would allow to establish global budgets for riverine C export prior to human impact. Ecosystem complexity helps to retain and rework organic materials (Gurnell et al. 2000). Therefore, organic matter transformations in this semi-natural, highly dynamic, and heterogeneous river system are much more efficient than in most of the regulated rivers. Detailed molecular characterization of freshwater and marine LMW DOM would be the most valuable way of choice to explain transformations and fates of riverine organic compounds. Many puzzles still need to be solved about the history of the global carbon cycle, but rivers are key, because the link the carbon cycle of the continents to that of the oceans.

**Literature cited**


Curriculum Vitae

1998 – 2002  Dissertation at the Swiss Federal Institute for Environmental Science and Technology (EAWAG), Switzerland

1996 – 1998  Graduate School at the University of Texas at Austin, USA
Marine Science Institute
(Marine Biogeochemistry and Microbial Ecology)

1988 – 1996  University of Vienna, Austria
1996 Completion of Master of Science
Department of Marine Biology
M. S. Thesis: *The net stimulatory effect of ultraviolet (UV) radiation on marine bacterioplankton*

1988 – 1996  Academy of Fine Arts, Vienna, Austria
1996 Completion of Diploma in Textile Arts
Department of Textile Arts and Tapestry
1992 Fuegerpreis
1991 Restoration work in Perak, Malaysia

1984 – 1988  Senior High School (Oberstufenrealgymnasium mit Instrumentalmusik
der Kreuzschwestern), Linz, Austria

1980 – 1984  Junior High School, Ottensheim, Austria

1976 – 1980  Primary School, Goldwörth, Austria

1970  Born in Linz, Austria