UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING, SCIENCE & MATHEMATICS

School of Ocean and Earth Science

DISSOLVED ORGANIC NITROGEN IN THE RIVER TEST AND ESTUARY.

by

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Thesis for the degree of Doctor of Philosophy

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Graduate School of the

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ABSTRACT

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Studies of nutrients in rivers and estuaries have generally focused on dissolved inorganic nitrogen (DIN), although this accounts for only a part of the total nitrogen (TN) in these systems. Dissolved organic nitrogen (DON) has not yet been fully considered as a factor contributing to the nitrogen pool in aquatic systems. Advances in the analytical determination and characterisation of DON have identified that it is potentially biologically available to phytoplankton and bacteria. Very few studies of DON have been conducted on UK rivers and estuaries therefore little is known of the concentrations, proportion of TN, seasonal variations and DON load entering estuarine waters from riverine sources.

An 18 month survey of the lower reaches of the River Test and upper estuary were conducted between July 2001 and December 2002 to investigate the temporal variation in nutrient concentrations. DON concentrations up to 152 µM were measured in saline samples and concentrations of up to 100 µM were recorded in freshwater samples from the River Test. DON was the second largest TN component, contributing up to 7 % of TN in the river and 13 % of TN in the upper Test estuarine waters.

DON showed no apparent relationship with season, flow or salinity, whereas dissolved organic carbon (DOC) concentration showed some seasonal changes. Concentrations of DON were reduced by tidal salt marshes and sewage treatment works were a source of DOC to the river. The estimated DON load entering the Test estuary in 2002 was 1.9×10^4 mol km^{-2} yr^{-1}, one order of magnitude lower than the nitrate load. The DON area-normalised load was in good agreement with other UK river systems.

The assimilation of different molecular size fractions of DON and DOC by bacteria was investigated in water collected from the lower reaches of the river. A combination of ultrafiltration and bacterial bioassays showed that the DON and DOC pools were made up of a range of molecular size classes but these did not appear to influence the growth of freshwater bacteria.

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DECLARATION OF AUTHORSHIP

I, Judith Melanie Homewood, declare that the thesis entitled Dissolved organic nitrogen in the River Test and estuary and the work presented in it are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
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Signed: ……..

Date:…….

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“I have never regretted what I did. I regret things I didn't do.”

Ingrid Bergman

LIST OF ABBREVIATIONS

Chl a	=	chlorophyll a
CRM	=	certified reference material
DIC	=	dissolved inorganic carbon
DIN	=	dissolved inorganic nitrogen
DOC	=	dissolved organic carbon
DOM	=	dissolved organic matter
DON	=	dissolved organic nitrogen
DOP	=	dissolved organic phosphorus
EA	=	Environment Agency
EEC	=	European economic community
EC	=	electrical conductivity
GANE	=	Global nitrogen enrichment project
GF/F	=	glass fibre filter
HAB	=	harmful algal bloom
HTCO	=	high temperature catalytic oxidation
HMW	=	high molecular weight
HMS	=	harmonised monitoring scheme
IPB	=	indophenol blue
IRGA	=	infra red gas analyser
LMW	=	low molecular weight
MW	=	molecular weight
NMW	=	nominal molecular weight
NVZ	=	nitrate vulnerable zones
OPA	=	orthophthaldialdehyde
PAR	=	photosynthetically available radiation
POC	=	particulate organic carbon
PON	=	particulate organic nitrogen
POM	=	particulate organic matter
Q	=	river flow
SOC	=	Southampton Oceanography Centre
STW	=	sewage treatment works
TDN	=	total dissolved nitrogen
TN	=	total nitrogen
TOC	=	total organic carbon
TON	=	total oxidised nitrogen
UWWT	=	urban waste water treatment
VHMW	=	very high molecular weight
WHO	=	World health organisation

1 CHAPTER ONE. INTRODUCTION

1.1 NITROGEN CYCLE

1.1.1 Significance of nitrogen in aquatic ecosystems

Nitrogen was discovered in the late 18th century but its importance as a limiting nutrient in plant productivity was not recognised until the mid 19th century by Liebig (1903-1973) (Galloway *et al.*, 2002). It is considered to be one of the most important nutrients as it is a major component of amino acids, which are needed to build proteins (Hornung, 1999). In aquatic environments nitrogen is generally considered to be the limiting nutrient in seawater, but there is controversy over whether this is an oversimplification of complex biogeochemical cycles and in certain marine environments phosphorus may limit production (Rabalais, 2002). The Redfield ratio indicates that the crucial ratio of nitrogen: phosphorus : carbon required for phytoplankton growth is 16:1:106 (Redfield, 1958). This is accepted as a fundamental principle in marine sciences, although it only considers the nutrient status of algae and not detrital matter, bacteria and zooplankton which may also be present (Hessen, 1999).

Atmospheric sources of nitrogen are fixed by leguminous plants, lightning, nitrifying bacteria or blue-green algae (Schlesinger, 1997; Capone, 2001; Galloway and Cowling, 2002). These fixation processes remove molecular nitrogen (N_2) from the atmosphere and break the triple bond joining the nitrogen atoms to combine them with hydrogen to form ammonium, which is biologically available to plants and animals (Herbert, 1999; Vitousek, 2002). The fixed nitrogen can be combined with oxygen to form nitrite (NO_2^-) and nitrate (NO_3^-) or alternatively a combination of carbon and hydrogen atoms to form organic molecules through microbial transformations, which transfer nitrogen through a series of redox states (Figure 1.1) (Schlesinger, 1997). Denitrifying bacteria (usually under anaerobic conditions) carry out the final step in the cycle and return nitrogen to the atmosphere as either nitrous oxide or N_2 (Herbert, 1999).

Although almost 80 % of the atmosphere is composed of nitrogen, fixation by natural means was historically limited by the need for large amounts of energy to break the N≡N bond (Breviere, 2000; Galloway and Cowling, 2002). The discovery of the Haber-Bosch process in 1913 using high temperature and pressure made it possible for humans to fix

atmospheric nitrogen into a biologically useable form (Equations 1.1 and 1.2) (Schlesinger, 1997; Galloway *et al.*, 2002).

$$3CH_4 + 6H_2O \rightarrow 3CO_2 + 12H_2 \qquad \text{(Equation 1.1)}$$

$$4N_2 + 12H_2 \rightarrow 8NH_3 \qquad \text{(Equation 1.2)}$$

The ammonia produced from the Haber-Bosch process was primarily used to manufacture ammonium fertilisers, which are soluble in water and form ammonium ions. The increased use of fertilisers led to higher rates of food production and consequently rapid population growth and waste production (Hessen, 1999; Van Breemen, 2002).

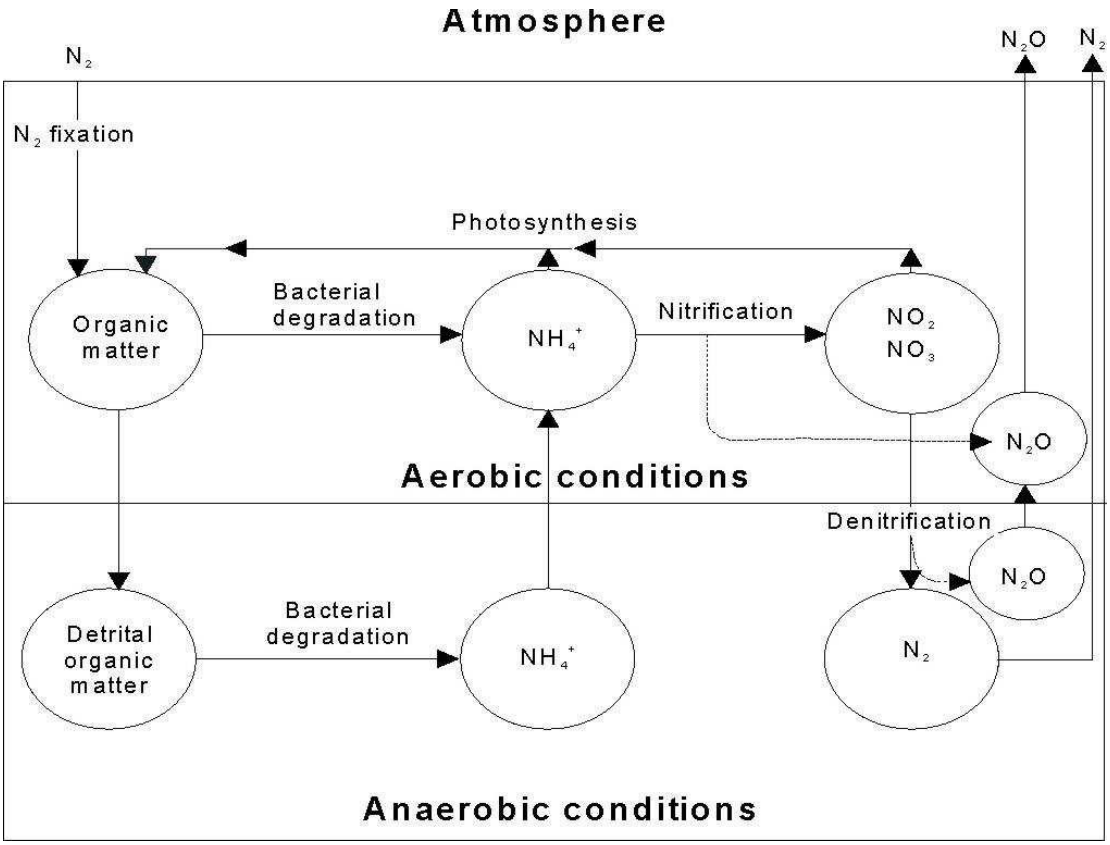

Figure 1.1. Microbial transformations in the Nitrogen Cycle (Schlesinger, 1997 adapted from Wollast, 1981)

1.1.2 Current trends and implications of the global nitrogen budget

Trends Since the Second World War anthropogenic fixation of nitrogen has increased dramatically (Galloway *et al.*, 1995; Neal, 2001; Rabalais, 2002). Approximately 165 Tg N yr^{-1} is fixed by human activities, of which 120 Tg N yr^{-1} is due to industry using the Haber-Bosch process for food production. Although only 20 Tg N yr^{-1} of this is actually ingested, 25 Tg N yr^{-1} is released from the combustion of fossil fuels, and 20 Tg N yr^{-1} are produced from other uses (e.g. legume crops)(Galloway and Cowling, 2002; Galloway *et al.*, 2002). This production equates to more anthropogenic fixation of reactive nitrogen than occurs through natural biological fixation (90 Tg N yr^{-1}) (Vitousek, 1994). Developed countries reduced the use of fertilisers in the 1990's recession, but in the longer term it is likely that increasing demand for food production in developing countries will elevate further the rates of anthropogenic fixation of nitrogen (Nixon, 1995).

Increases in reactive nitrogen over the last century have contributed to sustaining high food production to feed the growing human population (Cook, 1999; Tappin, 2002). Only a small proportion of anthropogenically fixed nitrogen is ingested, as nitrogen is readily lost from the environment (Galloway *et al.*, 2002; Vitousek, 2002). Much of the nitrogen in fertilisers thus enters water courses through runoff and groundwater flow (Black *et al.*, 1993; Heathwaite and Johnes, 1996). Even the nitrogen that is consumed by plants or animals, may enter rivers and estuaries through waste disposal (Wright, 1980; Nixon, 1995; Smith *et al.*, 1999). Despite recent policy changes to reduce the use of fertilisers, concentrations of nitrate entering aquatic systems remain high due to groundwater storage effects (Garcia-Ruiz *et al.*, 1998; Neal, 2001).

In addition to fertilisers, other anthropogenic increases in reactive nitrogen include the burning of fossil fuels, which releases nitrogen to the atmosphere in the form of nitrous oxide (Mosier *et al.*, 2002). This atmospheric nitrogen source returns to terrestrial and aquatic ecosystems via wet and dry deposition (Cornell and Jickells, 1999; Smith *et al.*, 1999; Cape *et al.*, 2001). In open oceans, atmospheric inputs of nitrogen can be important, whereas the majority of nitrogen transported to coastal oceans by rivers is terrestrially derived (Schlesinger, 1997). These rising levels of nitrogen inputs can increase production of fish biomass and survival rates in coastal waters (Rabalais, 2002). The greatest impact

is observed in nitrogen-limited ecosystems where the increases in nitrogen supply exceed the threshold for algal assimilation rates (Rabalais, 2002; Vitousek, 2002).

Implications Elevated nutrient loads entering aquatic systems have the greatest detrimental impact in areas of high productivity such as estuaries and coastal waters which have high species diversity (Shaw *et al.*, 1998; Smith *et al.*, 1999). Despite this, research on excess nutrients initially focused on lakes and freshwater environments (Nixon, 1995). Estuaries receive the largest nitrogen inputs from rivers, which in turn receive the majority of nitrogen from agricultural runoff (Tappin, 2002). Numerous definitions have been given to this phenomenon of degraded water quality, although many have been criticised as describing the effects rather than the process of eutrophication (Nixon, 1995). The definition given by Nixon (1995) states that eutrophication is "an increase in the rate of supply of organic matter to an ecosystem" which is appropriate since it is the accumulation of organic matter rather than inorganic nutrients that are frequently the cause for concern.

Freshwater and estuarine systems affected by nutrient enrichment, experience increased ecosystem productivity with consequent oxygen deficiency (hypoxia or anoxia), often resulting in increased turbidity and loss of submerged vegetation (Rabalais, 2002). The higher nutrient concentrations have been linked to the increased occurrence of harmful algal blooms (HABs) which are blooms of a single species that have the potential to cause health effects associated with increased toxicity in fish and shellfish (Paerl, 1988; Berg *et al.*, 1997; Hornung, 1999). These food web shifts (Vitousek, 1994) have disrupted the functioning of ecosystems by reducing biodiversity (Johannessen and Dahl, 1996) and caused habitat loss (Rabalais, 2002). Direct impacts on humans have included restrictions on irrigation and recreational usage as well as a reduction in water potability. Elevated concentrations of nitrate may have health implications for the increased occurrence of infantile methaemoglobinaemia, therefore the World Health Organisation (WHO) has set guidelines of 50 mg l^{-1} for nitrate concentrations in drinking water supplies (Anonymous, 1983; Hornung, 1999).

Atmospheric changes in reactive nitrogen have also impacted on terrestrial and aquatic ecosystems. The increased N_2O (nitrous oxide) emissions from combustion of fossil fuels (from both industry and increased usage of private transport) can influence climate change as N_2O is a greenhouse gas capable of destroying stratospheric ozone, which in turn adversely effects natural ecosystems (Schlesinger, 1997; Galloway *et al.*, 2002).

Atmospheric inputs of N_2O have also contributed to the acidification of freshwater lakes and rivers (Hornung, 1999).

Elevated levels of primary productivity, caused by an increase in reactive nitrogen may influence the global carbon cycle. Increased rates of carbon sequestration act as a carbon sink by storing large amounts of carbon in oceanic sediments (Delwiche, 1981; Smith *et al.*, 1999; Capone, 2001). If this is the case, eutrophication may actually be dampening the increase of the potent greenhouse gas carbon dioxide (CO_2) (Hopkinson *et al.*, 1993; Cornell *et al.*, 1995). There is much controversy over the importance of this process and whether a threshold level of CO_2 draw down may be reached (Fan *et al.*, 1998). This process highlights the intimate linking of the nitrogen and carbon cycles and emphasizes how perturbation in one biogeochemical cycle can have implications for others (Van Breemen, 2002).

Environmental monitoring of nitrogen loading European Community (EC) directives and international agreements (e.g. OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic and North Sea Conference Declarations) have been put in place to reduce the flux of nitrate (the most abundant form of inorganic nitrogen) from the land to the sea (Hydes, 2000; Nedwell *et al.*, 2002). The Urban Waste-Water Treatment (UWWT) directive (91/271/EEC) specifies the treatment standards for effluents entering water courses, with a particular focus on nutrient sensitive areas (Nedwell *et al.*, 2002). In contrast the Nitrate Directive (91/676/EEC) uses the designation of nitrate vulnerable zones (NVZ) to reduce ground water nitrate concentrations and manage land use change (Anonymous, 1999; Cook, 1999). Zones tend to be located in areas of extensive arable farming, or immediately upstream of large surface water abstraction points (Cook, 1999). Nitrate sensitive areas are also designated (by the UWWT directive) where NVZ receive large inputs from sewage treatment works (STW) and therefore fail to meet nitrate standards. In these areas water companies must increase the level of sewage treatment so that nitrate levels are reduced to acceptable levels.

The Harmonised Monitoring Scheme was established in the 1970's to study long term trends in nitrate concentrations across the UK as well as to fulfil international and EC obligations (Simpson, 1980). However, these agreements do not specify a requirement for the monitoring of organic nitrogen in surface waters, even though they required monitoring of nitrate, nitrite and ammonium concentrations (Anonymous, 1983). Without a national

network of long term measurements of DON in surface waters it is not possible to establish temporal variations in DON concentrations over different timescales.

1.2 DISSOLVED ORGANIC NITROGEN (DON) IN AQUATIC ECOSYSTEMS

1.2.1 Background and context

Most studies of nutrient inputs to rivers and estuaries have concentrated on measuring dissolved inorganic nitrogen (DIN) including nitrate (NO_3^-) nitrite (NO_2^-) and ammonium (NH_4^+). These inorganic forms of nitrogen are rapidly assimilated by micro- and macroalgae as well as macrophytes, thereby contributing to eutrophication (Nixon, 1995; Sanders *et al.*, 1997). However, DIN accounts for a part of the total nitrogen inputs to these aquatic systems and organic nitrogen (both particulate, PON, and dissolved, DON) has largely been ignored even though it is known to contribute to estuarine nitrogen loading (Seitzinger and Sanders, 1997b; Tappin, 2002). DON can vary between 20 – 50 % of the total dissolved nitrogen (TDN) content. Despite its dominance in many aquatic systems, little is known about its cycling and abundance and point source inputs (Seitzinger and Sanders, 1997b; Bronk *et al.*, 2000).

The reasons why DON has historically been neglected can be attributed to measurement difficulties and the assumption that DON is not biologically available to bacteria and phytoplankton (Antia *et al.*, 1991; Seitzinger and Sanders, 1997b). However, some studies have found that DON has the potential to stimulate the growth of harmful algal blooms (Paerl, 1988). DON is operationally defined as the dissolved nitrogen that is able to pass through a 0.45-1.0 μm filter (Lee and Fuhrman, 1987; Fuhrman, 1992; Hedges, 2002). There is some controversy over the precise poresize of filters as pores >0.2 μm will permit bacteria and viruses to pass through, although larger pores do remove the majority of particulate matter (Kaplan, 1994; Powell *et al.*, 1996).

The composition of DON remains largely unknown but in oceanic waters up to 50 % of the compounds have been identified (Sharp, 1983). The greatest fraction (less than 25 % of total DON) comprises hydrolysable amino acids, which can be subdivided into combined and dissolved free amino acids (Sharp, 1983). The second largest fraction (comprising up to 10 %) is urea, with the remaining constituents identified as vitamins, creatine, humic

and fulvic substances, nucleic acids (e.g. RNA and DNA) and amino sugars (Sharp, 1983; Walsh, 1989; Hopkinson *et al.*, 1993; Bronk, 2002). The preferential determination of DIN has been attributed to the simplicity of these molecules in contrast to the complex size and molecular structures of DON. However, analytical developments now permit the more precise measurement of DON in aquatic systems (Seitzinger and Sanders, 1997b).

DON is one of three major dissolved organic pools. These are dissolved organic carbon (DOC), phosphorus (DOP) and nitrogen (DON). Collectively these are known as dissolved organic matter (DOM) (Bronk and Ward, 1999; Hansell and Carlson, 2002). The importance of organic matter is increasingly being recognised and was incorporated into Nixon's (1995) widely acknowledged definition of eutrophication. Research into DON lags behind that of DOC mainly due to analytical difficulties such as lower concentrations and the fact that it cannot be measured directly (Bronk, 2002). Recent advances in the accuracy and precision of analytical techniques for measuring DON now allow this hitherto "missing" component of the nitrogen cycle to be included in nutrient budgets of aquatic ecosystems (Hopkinson *et al.*, 1993; Mortazavi *et al.*, 2001).

River and estuarine environments are important for the transportation of nutrients from terrestrial to coastal environments as well as being areas of rapid nutrient transformation. Few studies of DON concentrations have been carried out in UK rivers and estuaries. One of the first DON studies determined concentrations in upland Welsh streams to be between 4 and 5 µM (Reynolds and Edwards, 1995). Further research has found typical DON concentrations in UK rivers to be less than 100 µM (Edwards *et al.*, 1996; Russell *et al.*, 1998; Chapman *et al.*, 2001). Limited studies have been carried out on DON concentrations in UK estuaries, although two studies on Southampton Water have reported DON concentrations up to 178 µM (Hydes and Wright, 1999; Vigus, 2000). As higher concentrations tend to be measured in rivers and estuaries than in the coastal ocean (Bronk, 2002), a greater understanding of the sources and removal processes are needed in these areas of intense biogeochemical activity to give a clearer understanding of eutrophication (Seitzinger and Sanders, 1997b; Pakulski *et al.*, 2000; Mortazavi *et al.*, 2001).

1.2.2 Sources of DON

DON can originate from either autochthonous (internally produced) or allochthonous (imported) sources (Bronk, 2002). Many of the allochthonous sources are thought to be

similar to the DIN sources listed above, such as atmospheric and terrestrial inputs to aquatic systems (Figure 1.2). Meybeck (1993) estimated that 70% of TN entering coastal oceans from rivers is composed of DON whilst Cornell *et al*., (1995) suggested that equal amounts of DON are derived from atmospheric and riverine sources.

Incubation experiments indicate between 45 – 65 % atmospheric and 40 – 72 % riverine inputs of DON are bioavailable to bacteria and phytoplankton (Seitzinger and Sanders, 1997a; Seitzinger and Sanders, 1997b). The processes involved in the release and utilisation of DON in marine systems are presented in Figure 1.3. Release of DON from autochthonous sources include:

- passive diffusion from both bacteria and phytoplankton (including nitrogen fixers) (Bronk and Glibert, 1993; Bronk *et al.*, 1998)
- hydrolysis (of particulate matter) by bacterial exoenzymes (Berman *et al.*, 1999)
- exudation by phytoplankton (Antia *et al.*, 1991; Bronk *et al.*, 1994)
- grazing and excretion due to physiological stress (Bronk and Ward, 1999)
- viral induced cell lysis (Carlson, 2002)
- direct release from macroalgae, diagenesis of sediments (Burdige and Zheng, 1998)
- dissolution of detrital particles and micro and macrozooplankton faecal pellets (Fuhrman, 1992; Bronk, 2002).

1.2.3 Sinks of DON

The main sinks for DON in aquatic ecosystems include biotic (heterotrophic and autotrophic) uptake as well as abiotic (photochemical degradation and sorption of DOM onto particles)(Figure 1.3). Heterotrophic bacteria are able to utilise small organic compounds using extracellular hydrolysis to make amino acids and ammonium available for uptake (Antia *et al.*, 1991; Berman *et al.*, 1999). The mechanisms for autotrophic utilisation of DON remain unresolved, although there is potential for direct incorporation of DON using cell surface enzymes as well as direct phytoplankton utilisation of the products of heterotrophic proteolytic activity (Berman *et al.*, 1999; Bronk, 2002).

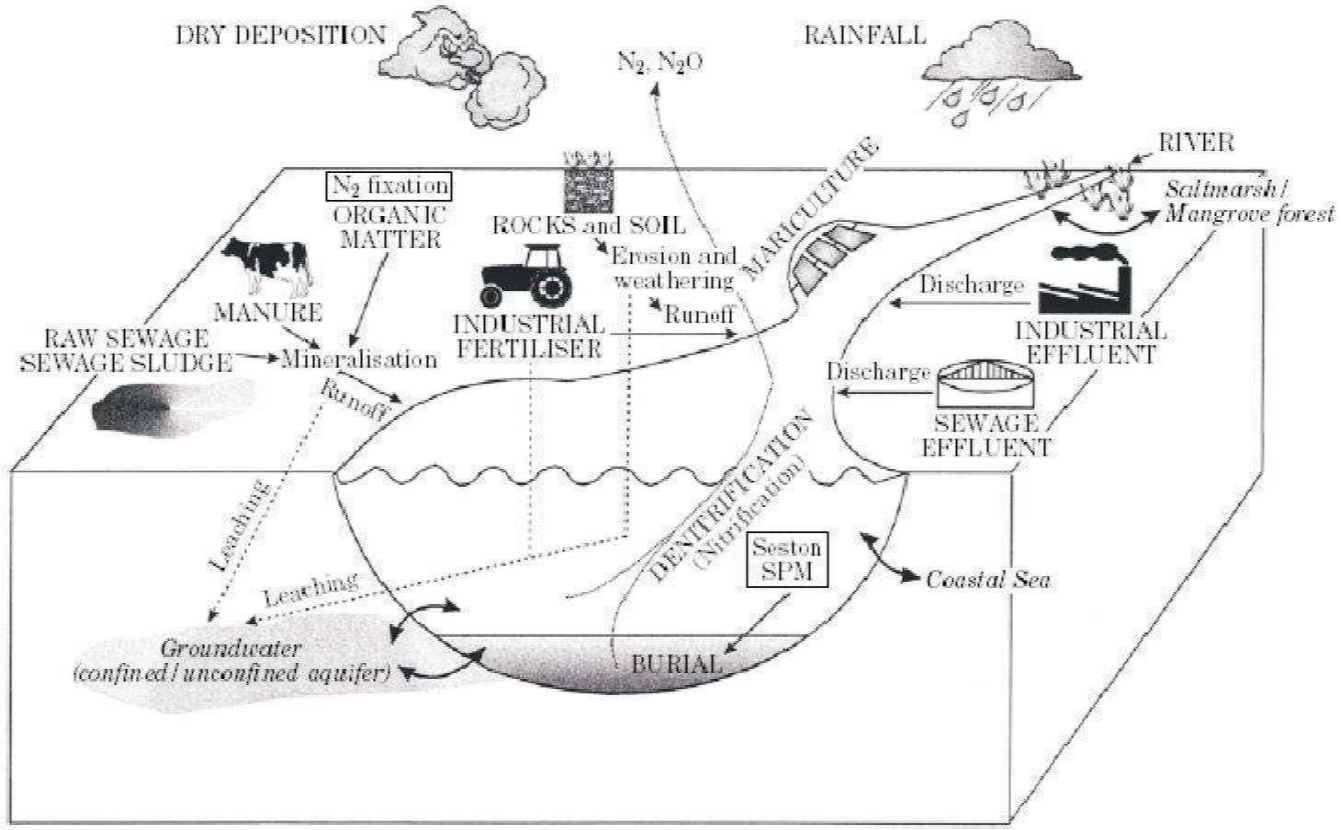

Figure 1.2. Estuarine sources and sinks of nitrogen. Interactions with coastal oceans are dependant upon tidal cycling and anthropogenic dredging activity (Tappin, 2002).

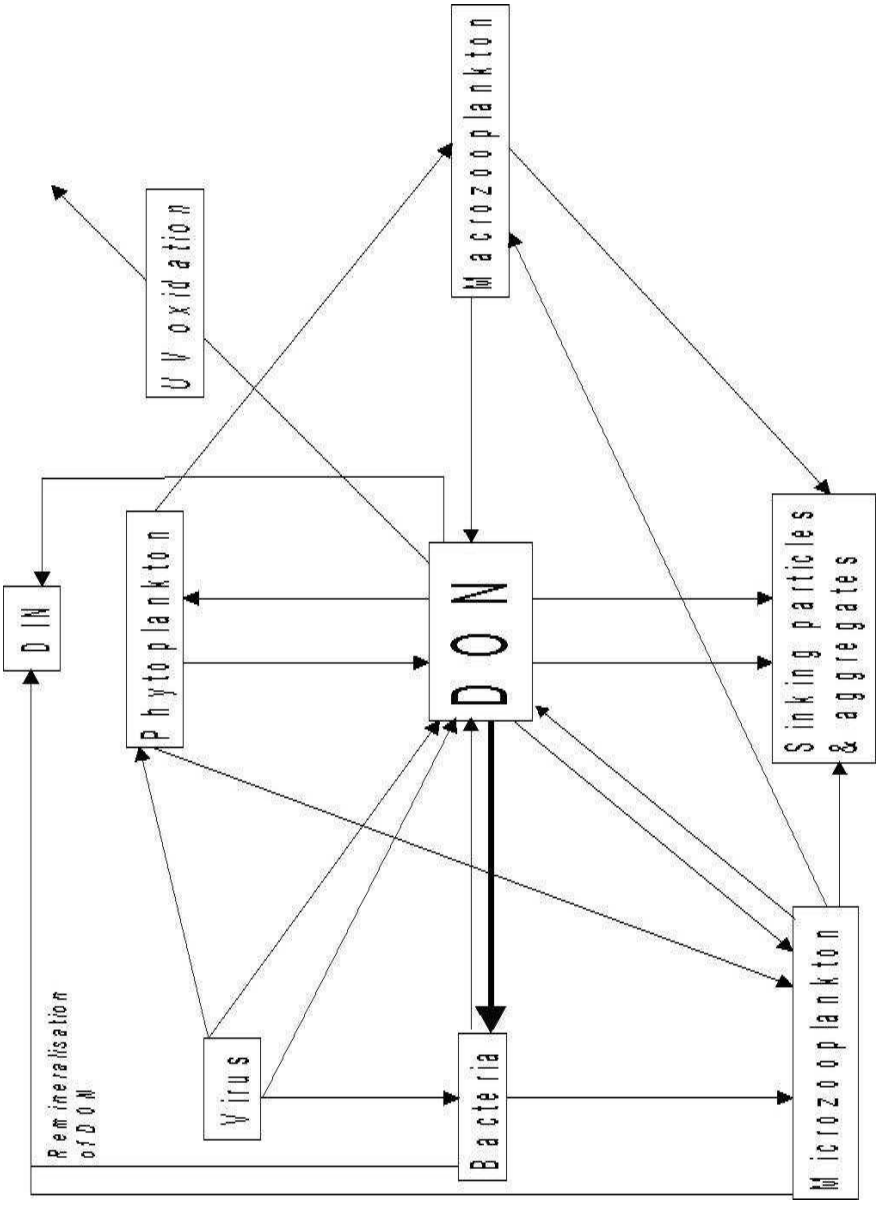

Figure 1.3. Schematic diagram of processes involved in the release and utilisation of DOM in aquatic systems (adapted from Carlson, 2002). The bold arrow indicates the dominant removal process. The dotted lines show viral infection causing cell lysis.

Much of the earlier culture work on autotrophic utilisation was carried out using higher concentrations of nitrogen than are available in the environment (Bronk, 2002). However, more recent culture work indicates that at natural concentrations of nitrate and ammonium, bacteria are able to out compete phytoplankton (Hagström *et al.*, 1984; Bronk, 2002).

Exposure of DOM to natural sunlight can cause photochemical reactions that either produce compounds that are released to the atmosphere (e.g. CO, CO_2) or produce compounds that are biologically available to bacteria through remineralisation of DOM (Bushaw *et al.*, 1996; Mopper and Kieber, 2002).

Another process is photobleaching, which is believed to be important in shallow riverine and coastal environments where high riverine DOM is diluted with oceanic water containing lower concentrations of DOM (Mopper and Kieber, 2002). This process has the ability to produce by-products from the breakdown of DOM, which can increase the bioavailability and increase the bacterial uptake and growth efficiency. Although the process is not so important in estuarine environments where the UV optical depth is shallow relative to the surface mixed layer depth (Mopper and Kieber, 2002). The sorption of DOM onto sinking particles in open ocean environments has been proposed as a potential removal mechanism. However, the magnitude of this process in these and other environments remains unknown due to a lack of data (Carlson, 2002).

1.2.4 Variations of DON in rivers and estuaries

Despite the increasing recognition that DON in rivers and estuaries is quantitatively important, research on the spatial and temporal variation of DON is lacking (Mortazavi *et al.*, 2001). In fact measurements of DON in UK rivers and estuaries are scarce and have tended to be focused on upland catchments, even though DON in subsurface flow is an important component of reactive nitrogen (Edwards *et al.*, 1996; Heathwaite and Johnes, 1996; Chapman *et al.*, 2001).

Seasonal cycling of DON in river and estuarine systems remains largely unknown, despite its importance as an intermediate state between autotrophy and heterotrophy (Mortazavi *et al.*, 2001; Bronk, 2002). In some environments such as Chesapeake Bay, USA, DON has elevated concentrations in late spring and summer (Bronk *et al.*, 1998). This contrasting

seasonal variation between DON and nitrate was also observed in upland Scotland streams (Chapman *et al.*, 2001). A study of 7 Australian estuaries showed lower concentrations of DON between January and May/June, associated with dilution of DON during flood months (Eyre and Pont, 2003). In other environments such as the River Lena, which flows into the Arctic Ocean, concentrations of DON decrease during the summer months (Cauwet and Sidorov, 1996). Freshwater or estuarine studies have not indicated clear seasonal variations, although these have been observed in some open oceanic waters (Hansell and Carlson, 2001). The detailed time series of measurements (an eleven year study of the Western English Channel; Butler *et al.*, 1979) showed an inverse seasonal variation with nitrate, corresponding with the findings of Bronk (1998) and Chapman (2001). Temporal variation in DON is of great importance in river and estuarine systems to understand high estuarine productivity and fully quantify the nitrogen load entering the oceans.

There have been no spatially intensive studies of DON concentrations in UK river and estuarine systems, although some research has focused on comparing concentrations between rivers to assess the significance of land uses and in particular point sources of nitrogen to aquatic systems (Cook, 1999; Nedwell *et al.*, 2002). In upland Scotland research found no relationship between DON and percentage catchment peat cover (Chapman *et al.*, 2001). In Rous River, Australia, the land use and geology were identified as important factors affecting spatial variations in DON (Eyre and Pepperell, 1999). Estuarine studies of DON distribution have not consistently found spatial variations. Surveys of Chesapeake Bay (USA) identified that DON was both spatially and temporally uniform (McCarthy *et al.*, 1977). However, an increase in DON was observed seawards from the River Lena, in the Siberian Arctic, which was related to increased production in brackish and coastal waters relative to the river (Cauwet and Sidorov, 1996). A greater understanding of the spatial variations in DON is necessary to identify point and diffuse sources of DON entering surface waters.

1.3 BIOAVAILABILITY AND MOLECULAR SIZE COMPOSITION OF DOM

1.3.1 Bioavailability of DOM

Historically DOM was thought to be refractory and therefore not biologically available to phytoplankton and bacteria (Seitzinger and Sanders, 1997b). This assumption was based on the premise that DOC varied conservatively with salinity, suggesting absence of autochthonous removal processes (Mantoura and Woodward, 1983). Secondly, the molecular weight composition of DOM indicated a dominance of biologically refractory (unreactive) high molecular weight (HMW) compounds relative to bioavailable low molecular weight (LMW) DOM (Saunders, 1976). However, more recent research suggests that a large proportion of DOC in river and estuarine environments is available for utilisation by bacteria over time scales of days to weeks (Tranvik, 1990; Amon and Benner, 1994; Bronk, 2002). Few studies have been carried out on the fate of nitrogen in DOM. Preliminary investigations suggest that DOM is more biologically reactive than originally thought which has important implications for higher trophic levels as DOM enters the microbial loop (Azam *et al.*, 1983; Bronk and Glibert, 1993; Amon and Benner, 1994; Bronk *et al.*, 1994). Studies on the Delaware and Hudson Rivers, USA, showed that 40 to 72 % of riverine DON was biologically available to estuarine bacteria (Seitzinger and Sanders, 1997b), and 20 to 55 % from boreal streams during spring flood was available (Stepanauskas *et al.*, 2000). A better understanding of DON bioavailability is needed to define the local and global implications of escalating inputs of anthropogenically fixed nitrogen and assess the contribution of DON to eutrophication of surface waters and the growth of harmful algal blooms (Seitzinger and Sanders, 1997b).

An increase in research into marine DOM concentrations and bioavailability (Hedges, 2002) was instigated by the publication of two papers reporting the determination of TDN using the High Temperature Catalytic Oxidation (HTCO) method (Suzuki and Sugimura, 1985; Sugimura and Suzuki, 1988). These authors suggested that oceanic concentrations of DOM were much higher than measured previously and proposed that the majority of respiration in the upper 1000 m of the water column was supported by oxidation of DOM, rather than sinking particulate organic matter (POM). An important finding was that DOM contained a much higher proportion of HMW molecules than thought previously, indicating an inability of previous analytical techniques to oxidise and measure these HMW molecules. The implications of these much higher concentrations of both DON and

therefore HMW molecules had implications for the molecular size composition and bioavailability of DOM. These two papers were subsequently withdrawn but stimulated research into analytical methods for determining concentrations of TDN as well as the cycling of DOM (Suzuki, 1993; Hedges, 2002).

1.3.2 Chemical characterisation of DOM

Without detailed information on the chemical composition of DON it remains unclear which characteristic of DON defines its bioavailability (Antia *et al.*, 1991). Studies have focused on determining the chemical composition by isolating specific components of DOM using solid phase extraction (either based on XAD resin or using nuclear magnetic resonance (NMR) spectroscopy; Aluwihare, 1999; Perdue and Ritchie, 2003) or investigating the distribution of molecular size of DOM using ultrafiltration (Ogura, 1974; Amon and Benner, 1996). Characterisation of DOM using molecular size fractionation has rarely been applied to river ecosystems (Meybeck, 1993; Amon and Benner, 1996), even though studies on humic and clear water lakes indicate that DOC is able to support bacterial growth in these environments (Tranvik, 1990). An assessment of the bioavailability of these molecular weight fractions may indicate the fate of riverine DOM, which is considered to be diagenetically young (< 150 years), generally highly degraded, and, in comparison with LMW DOM that has undergone extensive diagenetic change, rapidly remineralised prior to reaching the open ocean (Hedges *et al.*, 1994b; Benner *et al.*, 1995; Hedges *et al.*, 1997; Tranvik, 1998; Stepanauskas *et al.*, 1999a).

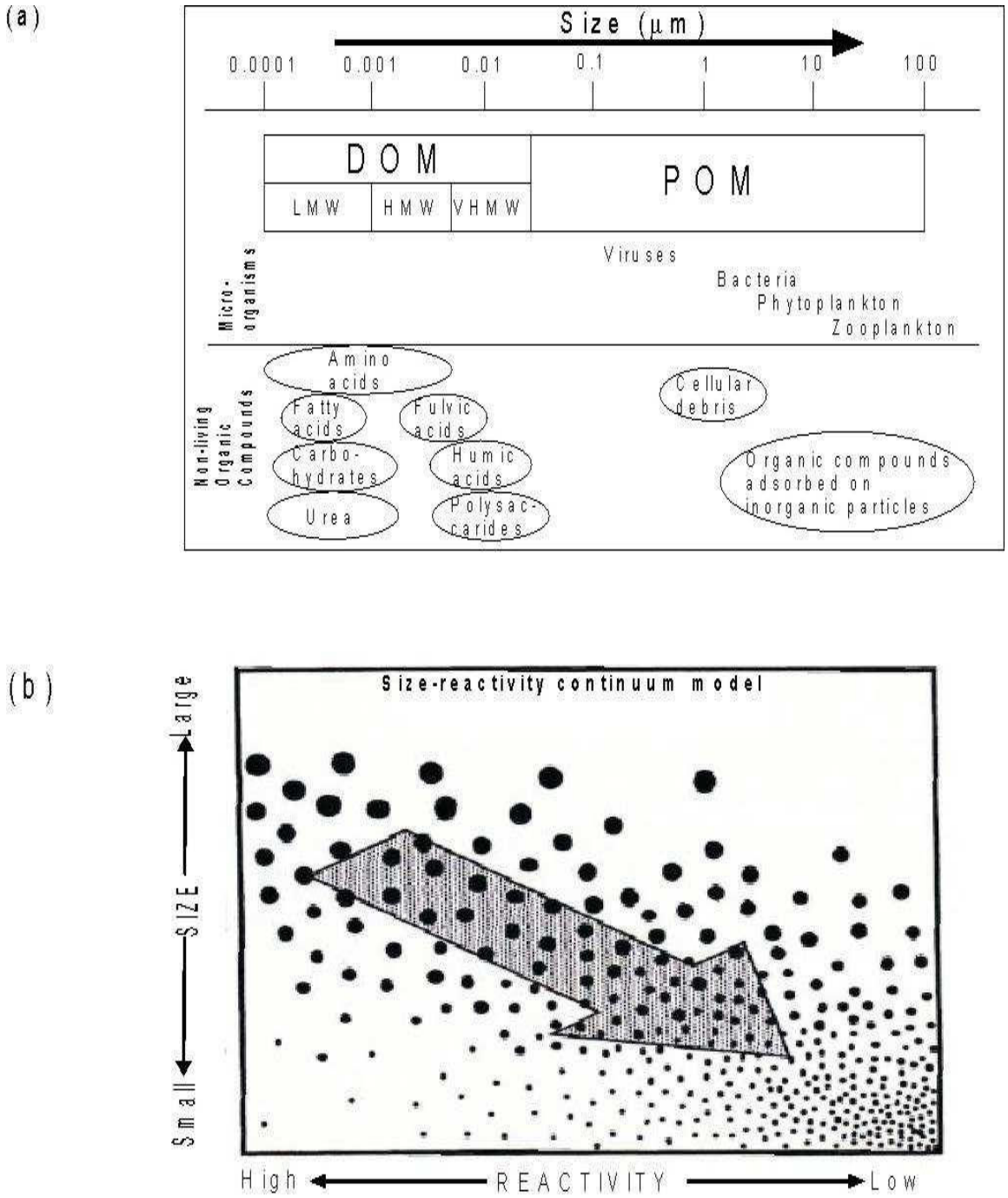

Figure 1.4. (a) Schematic of the molecular size continuum of living and non-living compounds (adapted from Buffle *et al.*, 1978 and Gough and Mantoura, 1990). (b) Conceptual model of the size-reactivity continuum indicating that HMW DOM is more reactive and therefore more bioavailable than LMW DOM (Amon and Benner, 1996). The relative molecular size of DOM is indicated by the size of dots with the largest being POM and the smallest LMW DOM. The arrow indicates the continuous change in degradation of organic matter from bioavailable POM and HMW DOM to refractory LMW DOM.

1.3.3 Size-reactivity continuum

The separation of DON into distinct size fractions can be carried out using ultrafiltration methods (Cheryan, 1986). Molecules greater than 1 kDa are considered to be HMW (e.g. amides, humic substances, polysaccharides etc.) and those smaller than 1 kDa are defined as LMW (e.g. urea, carbohydrates, dissolved combined amino acids (DCAA), dissolved free amino acids (DFAA), fatty acids etc.) (Figure 1.4a) (Spitzy and Leenheer, 1991; Benner *et al.*, 1997; McCarthy *et al.*, 1997; Bronk, 2002). Recently a third fraction named very high molecular weight (VHMW) DOM was introduced to describe particles greater than 30 kDa up to 0.2 μm (Mannino and Harvey, 2000). In the past LMW DOM (composed of simple organic molecules) was considered to decompose more rapidly than larger HMW molecules (Saunders, 1976). A more recent study suggests that bioavailable LMW makes up only a small fraction of DOM in natural waters (Amon and Benner, 1996). A new conceptual model for degradation of DOM was proposed. The size-reactivity continuum is based on the theory that HMW was more bioavailable as the components are newer and therefore diagenetically fresher than LMW (Figure 1.4b) (Tranvik, 1990; Benner *et al.*, 1992; Amon and Benner, 1994, 1996). Particulate studies in the Amazon River were used to extend the theory from the dissolved to the particulate phases of organic matter (Hedges *et al.*, 1994b). Other studies also support this size reactivity continuum, although Mannino and Harvey (2000) suggested that the model may depend on the structure of the organic matter. The importance of colloids in nitrogen aquatic research has not been fully researched (Powell *et al.*, 1996)

Most molecular size fraction and bioavailability studies have focused on DOC even though the utilisation of DOC, and DON can vary depending on the C:N of both the DOM and the utilising bacteria (Seitzinger and Sanders, 1997b). Bacteria have been used for DOM utilisation experiments because they have higher uptake rates as well as high surface area to volume ratio compared to phytoplankton, which are not necessarily able to take up all forms of DOM (Bronk, 2002). As bacteria are able to modify and remineralise DOM, they can exert a marked influence on the microbial food web (Benner *et al.*, 1995).
Since humic substances (HMW) comprise the largest fraction of DOM in most natural waters, freshwater bacterial bioassays have tended to focus specifically on these compounds (Thurman, 1985; Carlsson *et al.*, 1993; Carlsson *et al.*, 1999). Determining the

molecular size composition of riverine DOC and DON may elucidate the relative bioavailability of DOC and DON on the basis of molecular size.

1.4 CHARACTERISTICS OF THE TEST RIVER AND ESTUARY

The River Test in Hampshire is an aquifer-fed river with multiple interconnecting channels, draining approximately 1040 km^2 of primarily agricultural land and urban developments (Environment Agency, 2002; Nedwell *et al.*, 2002). The geology is predominantly Cretaceous chalk, particularly in the upper reaches of the catchment (Figure 1.5). Average annual rainfall is 824 mm and in the winter and autumn this rain recharges the groundwater aquifers giving relatively consistent flows throughout the year, with a mean annual river flow of 11.8 $m^3 s^{-1}$ (Environment Agency, 2002, 2004). The 64 km long river flows into its estuary at Redbridge, Southampton about 7 km upstream of the confluence with the Itchen estuary at Dockhead where it forms Southampton Water (Figure 1.6)(Environment Agency, 1999).

The estuary is both macrotidal and partially mixed and has a mean tidal range of 4.5 m, spring tide flushing rate of 26 hours and neap tide flushing rate of 76 hours (Wright *et al.*, 1997; Hydes, 2000). It has a double high water which occurs 2 hours apart and up to 2.5 km tidal excursion. Southampton Water drains the second largest catchment area on the South coast of England and contributes an important freshwater input to the English Channel (Nedwell *et al.*, 2002). The Environment Agency has an extensive water quality sampling programme throughout the Test River system, although this does not include measurements of DON concentration (Simpson, 1980).

The River Test was selected for this study in preference to the Itchen because a tidal barrier in the upper reaches of the Itchen estuary prevents the upstream propagation of tidal influence, which may have prevented the collection of samples over a wide salinity range. In contrast, the upper Test estuary has extensive reed bed communities and salt marshes, with no anthropogenic barriers to limit mixing of fresh and estuarine water (Hydes and Wright, 1999). The lower Test permits the sampling of the mixing zone between fresh and estuarine water needed to determine salinity-nutrient mixing.

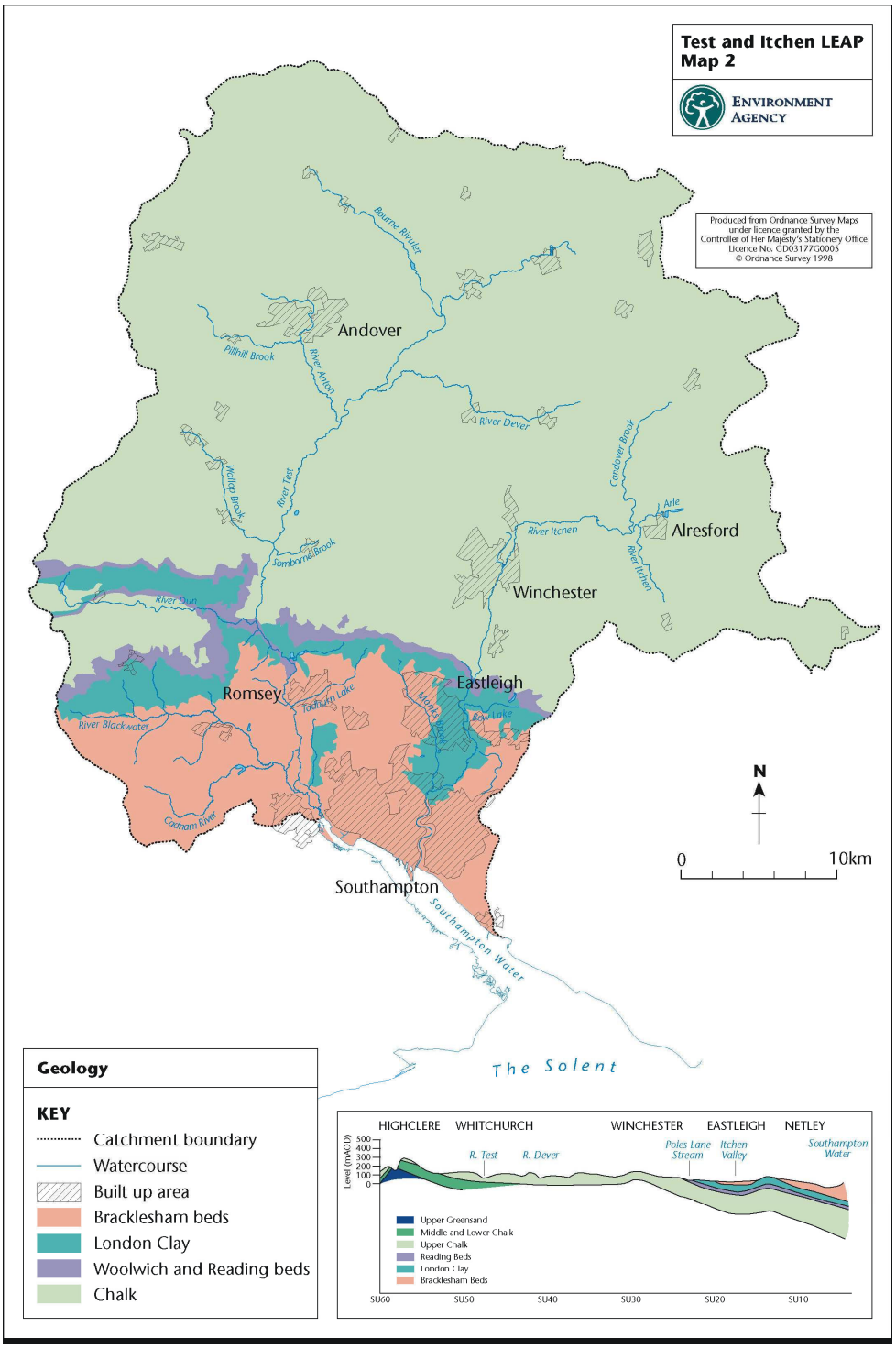

Figure 1.5 Geological map of the Test catchment, Hampshire, UK (Environment Agency, 1999).

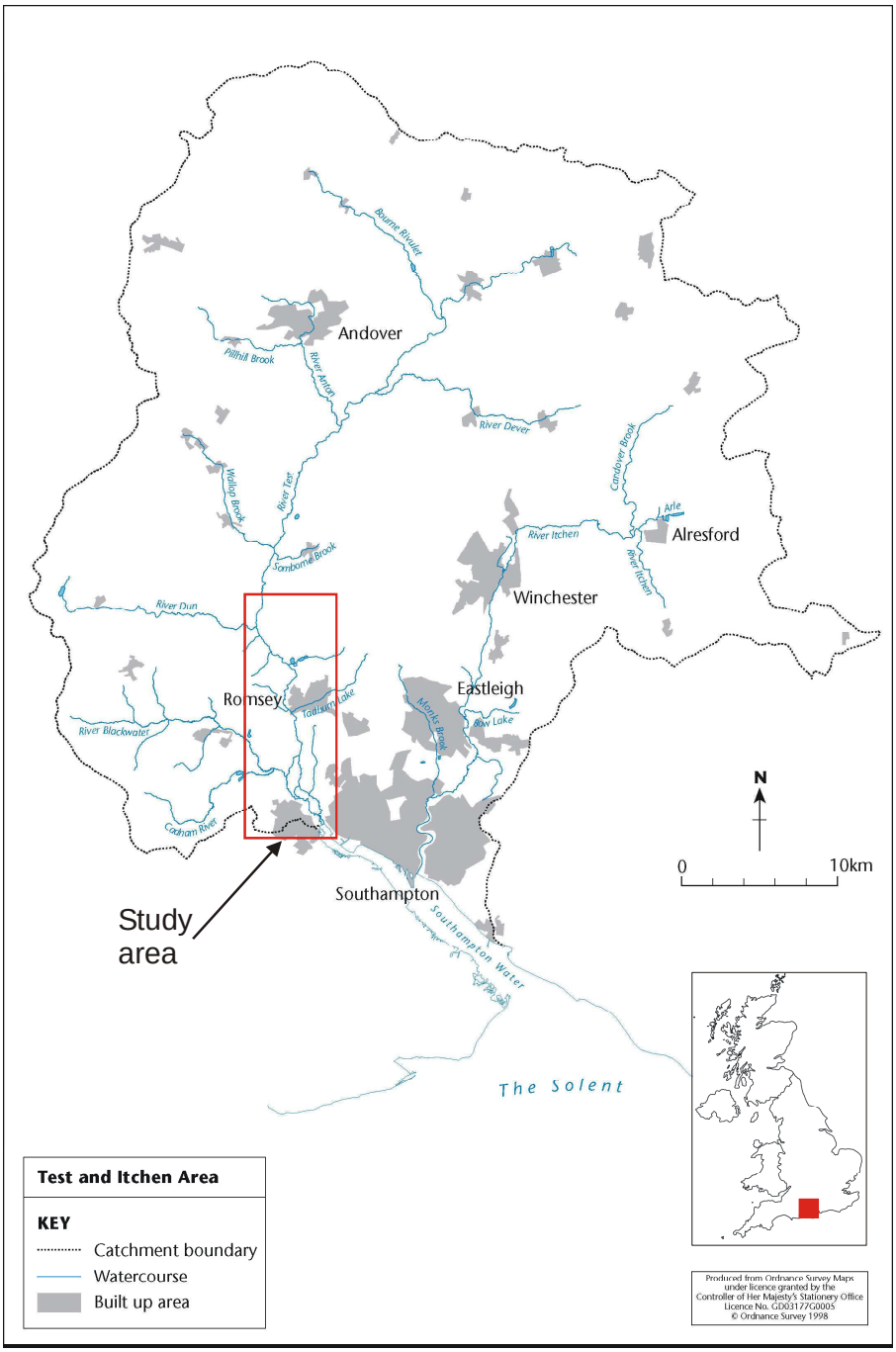

Figure 1.6. Rivers Test and Itchen draining into Southampton Water, Hampshire, UK (adapted from Environment Agency, 1999).

The river achieved an A grade for the general quality assessment carried out in 1998 (Environment Agency Pers. Comm., 2004). The river contains > 80 % saturation of dissolved oxygen (DO), < 2.5 mg l^{-1} biochemical oxygen demand (BOD) and < 0.25 mg l^{-1} ammonium (Environment Agency, 1999). The water percolates through the chalk and becomes purified therefore producing springs with a high clarity (Environment Agency , 2004). Several areas of the catchment, particularly in the lower River Test and upper estuary, are designated to protect the unique habitats, species and pastoral character of the valley (Environment Agency, 1999). Much of the study area is an EC designated freshwater (salmonid) fishery (Environment Agency, 1999).

The river contains high concentrations of nitrate throughout its length (>400 µM) and is a major source of nutrients to the Test Estuary and Southampton Water (Hydes, 2000). Nitrate is present in high concentrations in the chalk aquifer with more diffuse inputs leached from agricultural land. Long term increases in nitrate have been observed from previous studies from an average of 342 µM between 1974-1979, to 422 µM between 1990 and 1997 (Hydes and Wright, 1999; Arbuthnott, 2001). Ammonium inputs are assumed to be mostly from effluent point sources where secondary-treated sewage from a number of sewage treatment works (STW) enters the river system (Wright, 1980; Hydes, 2000). These increasing nutrient concentrations are a problem in lowland catchments in Southeast England, which have low rainfall and high evapotranspiration and there is less capacity to dilute nutrient inputs due to the lower summer baseflow (Neal *et al.*, 2000a). In 1994 the River Test was designated a nitrate sensitive area under the EC Urban Waste Water Treatment (UWWT) Directive. This designation identified that water companies needed to act to reduce the nutrients entering the river from STW to prevent the occurrence of algal blooms. Southampton Water is classified as hypernutrified, which although not perceived as a problem in itself, may be a precursor to eutrophication (Wright *et al.*, 1997; Hydes and Wright, 1999; Holley, 2002). The low turbidity in Southampton Water (due to the drainage from chalk catchments) means that light is not a limiting factor to planktonic primary production in the estuary (Balls, 1994; Shaw *et al.*, 1998). Seasonal variations in the Southampton Water nutrient levels have been reported in previous studies and reduced levels of nitrate and phosphate were associated with phytoplanktonic blooms (Kifle and Purdie, 1993; Iriarte and Purdie, 1994).

1.5 THESIS AIMS AND OBJECTIVES

The overall aim of the research was to determine temporal and spatial changes in DON in a high nitrate river-estuarine system and, in addition, to investigate the bioavailability of DOM relative to molecular size distribution.

The specific objectives of this research were:

- To determine the temporal variation in DOM in relation to dissolved inorganic nitrogen concentrations (nitrate, ammonium), particulate organic nitrogen and carbon in the lower reaches of the River Test and the upper estuary.
- To determine the contribution of DON to riverine nitrogen loads.
- To assess spatial variations in DON and DOC along the Test river / estuary continuum and identify the importance of point source inputs along the river and the influence of intertidal salt marshes on nutrient concentrations.
- To determine the molecular size composition of freshwater DON and DOC from the River Test and to investigate the bioavailability of different size fractions using a bacterial bioassay.

1.6 THESIS STRUCTURE

Chapter one (*Introduction*) includes a review of the nitrogen cycle and the current state of knowledge of dissolved organic nitrogen in aquatic environments. This includes a discussion of the existing research into spatial and temporal variations in riverine DON and its bioavailability. A description is given of the River Test and estuary and the thesis aims and objectives are presented. Chapter two (*Analytical Methodology*) describes the sample collection, preparation and storage protocols used in the field and laboratory to prepare samples prior to analysis. Details are given of the analytical procedures and development of the analysis protocol applied to the high temperature catalytic oxidation (HTCO) technique for determining DON concentrations. Temporal variations in DIN and DOM in the River Test and its estuary over seasonal and annual timescales are presented in chapter three (*Temporal variations in the inorganic and organic nitrogen in the River Test and its estuary*). Temporal variations in both the concentrations and proportions of TN from both freshwater and saline samples are discussed. Seasonal variations in the nitrogen load of the Test Estuary are compared with previous load calculations. Chapter four (*Spatial variations in inorganic and organic forms of nitrogen and carbon in the River Test –*

estuary) describes both the freshwater and estuarine spatial variations in DIN and DOM in the River Test-estuary. Point source effluent inputs downstream of a fish farm and sewage treatment works are investigated. Salt marshes located in the tidal reaches of the upper Test Estuary provide an interesting insight into nitrogen transformations. The ultrafiltration of water from the River Test and the calibration of the analytical technique using organic molecules of known molecular size are discussed in chapter five (*Assessing the chemical characterisation and bioavailability of riverine DON and DOC*). Data from bacterial DOM bioassays are presented and the implications for DOM utilisation discussed. Chapter six (*Conclusions and suggestions for further work*) summarises the main findings of the thesis and implications for the cycling of DON and DOC in a river / estuary system. Considerations for future research in these environments are discussed.

2 CHAPTER TWO. METHODOLOGY

2.1 SAMPLING STRATEGY

A sampling strategy was devised to collect water samples on a monthly basis over an eighteen month period (July 2001 to December 2002) to characterise both spatial and temporal variations in the lower River Test and upper Test estuary. The sampling dates were approximately every four weeks and were planned to coincide with spring tides wherever possible. The sampling dates and predicted tidal heights on those days are given in Appendix A. The sampling programme commenced at Redbridge on the upper Test estuary during low tide and aimed to sample both main and parallel river channel sites over a 16 km transect upstream to Mottisfont, north of Romsey (Figure 2.1). The final samples were collected later in the day from several sites in the upper Test estuary including revisiting Redbridge.

A detailed description of each sampling location and distance from Redbridge of each sampling point is given in Table 2.1 and presented on a map of the study area (Figure 2.1). Multiple parallel channels in the tidal reaches of the lower Test meant it was not possible to fully characterise the water composition from just one sampling site. Therefore sites were selected on several channels to optimise the collection of water samples covering a range of salinities in the tidal marsh area.

Sample collection from the estuarine site at Redbridge Park did not commence until June 2002 when the site became accessible. Two samples were collected from Redbridge and Redbridge Park at high tide, one from the surface and the other towards the bottom of the water column using a Niskin sample bottle.

2.2 SAMPLING AND STORAGE PROTOCOL

2.2.1 Sample collection

The water temperature (Appendix B), conductivity (Appendix C) and salinity were recorded *in situ* at each sampling site using a WTW model LF597-S salinometer. Salinity measurements of collected water samples were repeated on return to the laboratory (Appendix D).

Site No.	Distance upstream from Redbridge (km)	Grid Reference	Surface / Bottom	Site Area	Site Description
18	-0.4	SU 3718 1343	Bottom	Upper estuary	Redbridge park; high tide sample collected from depth
17	-0.4	SU 3718 1343	Surface	Upper estuary	Redbridge park; high tide sample collected from the surface
16	0	SU 3700 1373	Surface	Redbridge	High tide sample collected from the surface
1	0	SU 3700 1373	Bottom	Redbridge	High tide sample collected from depth
2	0	SU 3700 1373	Surface	Redbridge	Low tide sample collected from the surface
6	0.6	SU 3693 1448	Surface	Test Lane lower	Salt marsh (divided channel)
6a	1.6	SU 3673 1498	Surface	Test Lane upper	Salt marsh (divided channel)
3	1.3	SU 3625 1420	Surface	Testwood	Tree lined banks, agricultural land (divided channel)
4	1.5	SU 3610 1448	Surface	Testwood	Rural, commercial fishing (divided channel)
5	1.5	SU 3615 1445	Surface	Testwood	Rural, commercial fishing (divided channel)
7	3.5	SU 3513 1555	Surface	Nursling Mill	Agricultural land (minor part of divided channel)
8	3.5	SU 3555 1578	Surface	Nursling Mill	Agricultural land (major part of divided channel)
9	4.0	SU 3540 1625	Surface	Broadlands Lake	Next to motorway. Enclosed by pasture land & fishing lakes
10	5.7	SU 3547 1786	Surface	Longbridge	Surrounded by agricultural land
11	8.9	SU 2068 3493	Surface	Romsey	Downstream of urban conurbation, upstream of STW inputs
12	15.3	SU 2563 3302	Surface	Kimbridge	Intense fish farming area (divided channel)
13	15.4	SU 2555 3293	Surface	Kimbridge	Intense fish farming area (major part of divided channel)
14	15.4	SU 2568 3323	Surface	Kimbridge	Intense fish farming area (divided channel)
15	16.0	SU 2643 3310	Surface	Mottisfont	Agricultural land both sides of the river

Table 2.1. Sites on the River Test and estuary sampled on a monthly basis from January 2001 to December 2002.

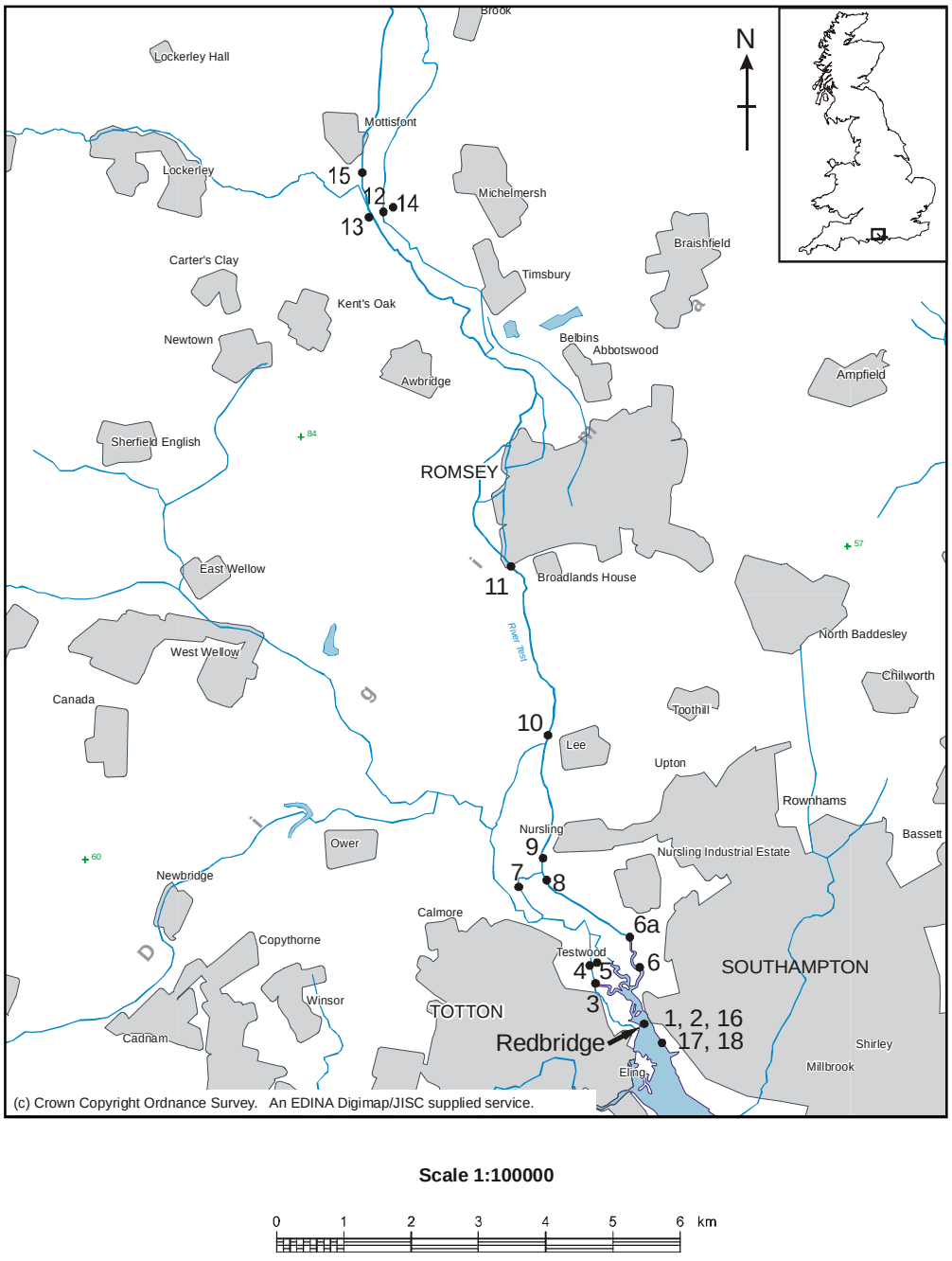

Figure 2.1. Map of all sites on River Test and estuary sampled on a monthly basis from January 2001 to December 2002. Grey shading indicates urban areas.

The water samples were collected just below the surface using an acid washed plastic bucket and transferred to rinsed 300 ml glass medicine bottles that had been previously combusted at 550 ^{o}C for 4 hours. These samples were stored for up to five hours in a cool box before returning to the laboratory for filtration and storage.

2.2.2 Filtration and sample storage

In the laboratory, 200 ml of each sample was filtered though Whatman GF/F (47 mm) filters (precombusted at 500 ^{o}C for 4 hours) using a Millipore all glass filtration system and hand vacuum pump. Filters were placed in petri-slides and stored frozen (at –20 ^{o}C) for later particulate organic carbon (POC) and nitrogen (PON) analysis. The filtrate from each sample was divided between a pre-combusted (550 ^{o}C for 4 hours) 100 ml glass bottle for later total dissolved nitrogen (TDN) analysis and a 20 ml plastic capped vial for later nitrate analysis. These samples were then stored frozen prior to analysis. 50 ml of water was filtered from the remaining sample though a Whatman 25 mm (diameter) GF/F filter using an in-line swinex filtration system. These filters were folded in half and frozen (at –20 ^{o}C) for later chlorophyll a (Chl a) analysis. The 50 ml of filtrate was stored at 4 ^{o}C in an acid washed glass bottle with 2 ml of alcoholic phenol for ammonium (NH_4^+) analysis (Parsons *et al.*, 1984).

2.3 ANALYTICAL PROCEDURES

The analysis of samples collected during the 18 month sampling survey was carried out using the techniques listed in Table 2.2. Samples from all sites were analysed for ammonium, nitrate + nitrite (TON) and chlorophyll a. Analyses of dissolved organic carbon (DOC), TDN and the filters for POC and PON was only conducted for mainstream sites (i.e. where the River Test was constrained within one channel, or the largest of several parallel channels containing the highest flow). A discussion of the sampling programme and selection of these mainstream sampling sites is presented in Chapter 4. Concentrations are presented in μM (± standard deviation) with the exception of chlorophyll a, which is measured in μg l^{-1}.

2.3.1 Nitrate

Nitrate was analysed following cadmium-copper reduction of nitrate to nitrite using a Bukard Scientific (Model SFA-2) segmented flow auto analyser, linked to an 80 cup autosampler with chart recorder and computer with microstream data capture software (Grasshoff *et al.*, 1983). The analysis is based on a chemical reaction between nitrite and sulphanilamide hydrochloride to form a diazonium salt. This in turn couples with N-(1-naphthyl)-ethylene-diamine dihydrochloride to produce an azo dye which can be quantified spectrophotometrically. Theoretically the technique measures total oxidised nitrogen (TON) as the cadmium reduction column converts nitrate to nitrite, but for the purposes of this study the term nitrate will be used to describe the combined measurement of nitrate and nitrite. Nitrite has not been considered separately as the proportion of nitrite in the TON is negligible; for example in March 2002 concentrations of nitrite ranged from 0.5 to 1.3 µM (i.e. <1 % TON). This observation was in agreement with water samples analysed by the Environment Agency (analytical procedures are discussed in §2.4).

Table 2.2. Nutrient analysis and storage procedures. OPA = orthophthaldialdehyde.

Determinand	Storage method	Analysis technique	Analytical Procedure
Nitrate & nitrite	Frozen at –20 ^{o}C	Autoanalyser (Burkard Scientific SFA-2)	Grasshoff *et al.*, 1983
Ammonium	Phenol at 4 ^{o}C	Indo-phenol blue method (U-2000 spectrophotometer)	Parsons *et al.*, 1984
Ammonium	None	OPA method (Chemlab continuous flow autoanalyser)	Kérouel and Aminot, 1997
DOC & TDN	Frozen at –20 ^{o}C	HTCO and pyro chemiluminescent nitrogen specific GC detector (Shimadzu TOC 5000A in line with Antek Model 705E)	Alvarez-Saldago and Miller, 1998
POC & PON	Frozen at –20 ^{o}C	Elemental analyser (Carlo Erba EA1108 elemental analyser)	Verardo *et al.*, 1990
Chlorophyll a	Frozen at –20 ^{o}C	Fluorometer (10 AU-Turner Design)	Parsons *et al.*, 1984

Standards were prepared at 20, 40, 60 and 80 µM using sodium nitrate and Milli-Q water. A ten times dilution of the water was required using 2 ml sample and 18 ml saline carrier stream solution (20 gl^{-1} sodium chloride) to measure nitrate using this technique. After mixing, the saline diluted river water was dispensed into three rinsed sample cups to give

replicate measurements of nitrate. The pipetting errors were determined to be < 3 %, therefore this dilution process may account for the greatest error in the determination of dissolved organic nitrogen (DON) by the difference method employed (Hopkinson *et al.*, 1993). Errors were thus minimised by using gravimetric dilution of samples. The cadmium column reduction efficiency was checked at the beginning of every analysis day using 60 µM nitrite standard. The column efficiency was consistently > 97%. The analysis had a mean detection limit of 1.2 µM based on three standard deviations of replicate measurements of the lowest standard (Miller and Miller, 1993). The mean standard deviation associated with three replicate analyses of one sample was 2 µM.

Storage stability The nitrate samples were stored frozen at –20 ^{o}C. Many studies have considered storage techniques and freezing is widely used for storing nitrate samples, although the precision of the analysis often decreases after storage (MacDonald and McLaughlin, 1982; Chapman and Mostert, 1990; Dore *et al.*, 1996).

Salt effect Measurements carried out across a wide range of salinities are known to incur a salt effect, especially in colorimetric analyses (Stewart and Elliott, 1996). The salt effect influences the absorbency and therefore the refraction of light in the detector cell, due to the formation of different reaction products (Stewart and Elliott, 1996). To minimise any salt effects a carrier solution of 20 gl^{-1} NaCl was used for all samples, although the majority of samples collected during these surveys had a zero salinity, therefore the salt effect was considered negligible (Wood *et al.*, 1967; Stewart and Elliott, 1996).

2.3.2 Ammonium (indophenol blue method)

The samples for ammonium analyses were stored in a refrigerator at 4 ^{o}C with alcoholic phenol, a reagent required for the indophenol blue (IPB) analysis method which is considered to preserve ammonium (Parsons *et al.*, 1984). The reaction to determine the ammonium concentration involves reacting 10 ml of sample preserved with 0.4 ml phenol (in 20 ml McCarthy bottles) with 0.4 ml sodium nitroprusside (prepared from 0.5 g in 100 ml of deionized water). Then 1 ml oxidising solution is added to each bottle. This is composed of a 4:1 of alkaline reagent (100 g of sodium citrate and 5 g of sodium hydroxide in 500 ml of deionized water) and sodium hypochlorite solution (1.5 N). The 20 ml McCarthy bottles were individually sealed with parafilm and left in the dark for 4

hours prior to analysis. The indophenol blue colour produced is quantified spectrophotometrically. Four analytical replicates from each reaction vessel were injected into a Hitachi U-2000 spectrophotometer using a sipper and absorbance measured using a 4 cm cell at 640 nm wavelength. The technique was very sensitive to contamination and frequently one of the four replicates was identified as an outlier. The detection limit for this technique (three times the standard deviation of replicate blank measurements) was 0.4 µM. All reagents were prepared using ammonium free water, which was produced by passing Milli-Q water through a column containing cation exchange resin. The standards (0, 1.5. 2.5, 5, 7.5, 10 µM) were diluted on the day of analysis with freshly prepared ammonium free water. The absorbance measured when the reagents were added to ammonium free water (i.e. blank) was equivalent to an ammonium concentration of < 3 µM.

Storage Stability Samples collected in the field were kept in a cool box for up to 5 hours before being filtered in the laboratory and preserved with alcoholic phenol. It was assumed that between collection, filtration and storage the ammonium concentration would not change. In order to test this assumption, triplicate samples were collected from three sites in the upper Test estuary, mid and upper River Test (sites 16, 9 and 15 on Figure 2.1) in December 2001 and March 2002. One set was filtered and stored with alcoholic phenol solution in the field, immediately after collection. The second set was processed in an identical manner back in the laboratory and a third set (only collected in March 2002) was not filtered and again stored with phenol on return to the laboratory (Figure 2.2). A consistently lower concentration of ammonium was recorded in the samples that were filtered and preserved immediately with phenol in the field (Holmes *et al.*, 1999). Higher concentrations were measured in samples stored with phenol in the laboratory regardless of the filtration procedure, except for the estuarine sample, which had markedly higher ammonium concentrations, possibly related to a higher particulate loading.

From May 2002 the above sampling protocol was adapted accordingly to preserve the ambient *in-situ* ammonium concentrations at each sampling site (i.e. the sample was filtered and preserved with phenol in the field immediately after collection) (Holmes *et al.*, 1999). The change in ammonium concentration between preserving in the field and the laboratory could be due to the volatile nature of ammonium. However, a decrease rather than increase was anticipated due to volatilisation or adsorption to the walls of the container (Kérouel and Aminot, 1997; Cape *et al.*, 2001).

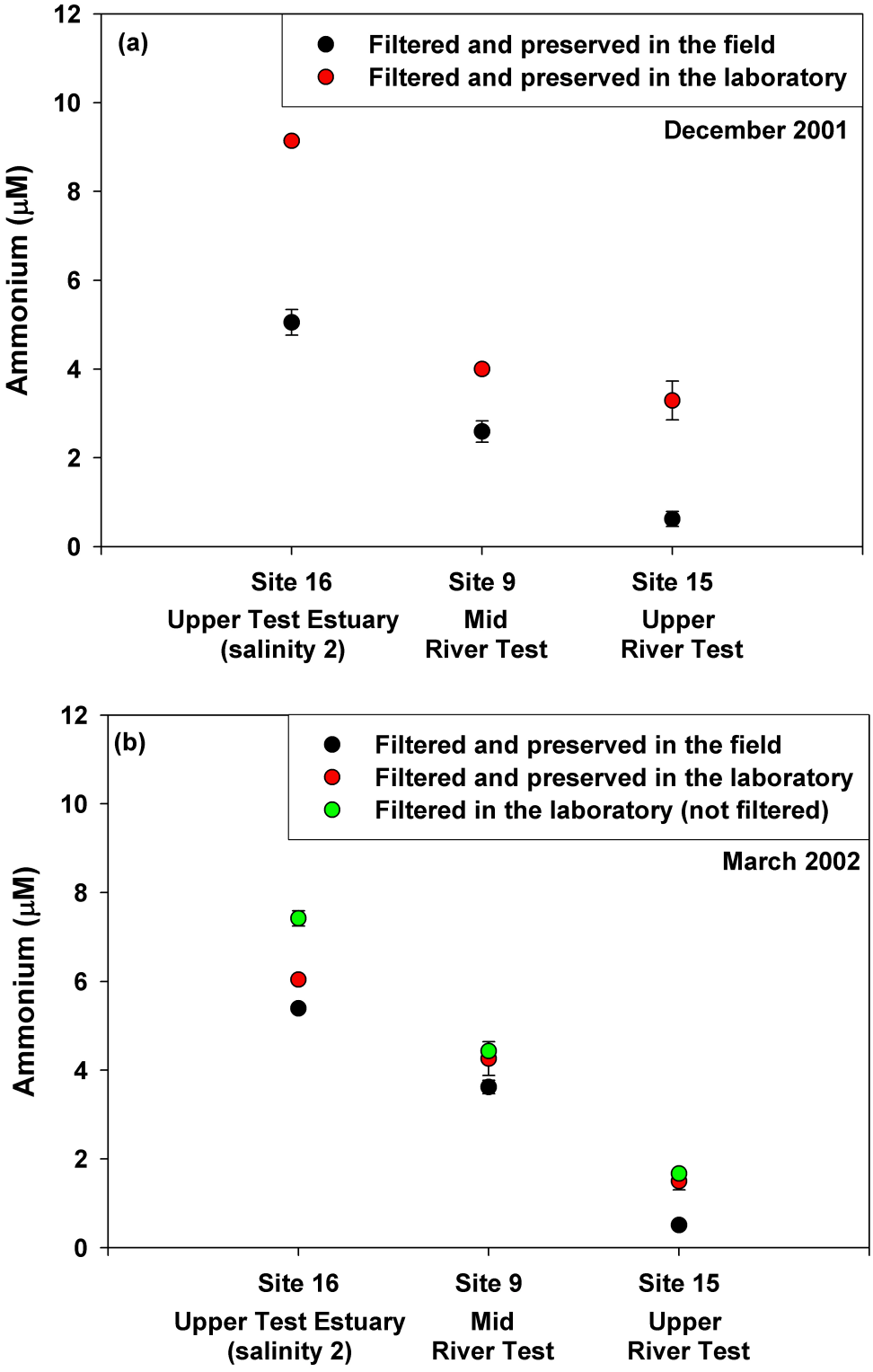

Figure 2.2. Experiment from (a) December 2001 and (b) March 2002 to compare filtration and preservation of ammonium samples in the field and laboratory. Where error bars are not shown, standard deviations (for up to 4 replicate measurements) are smaller than the size of the symbol.

The increase may be caused by acquisition of ammonium from the headspace prior to preserving with phenol. The effect of preservation on other determinands was also considered. Chlorophyll a (Figure 2.3a) and nitrate (Figure 2.3b) concentrations showed no difference between filtration in the field and laboratory. It was not practical to use the glass filtration system in the field for TDN samples.

Salt effect Salinity is known to not only influence the indophenol-blue reaction causing different reaction products, but also to change the pH of the reaction (Stewart and Elliott, 1996; Aminot *et al.*, 1997). An optimum pH of 10.5 was suggested for this technique, although the addition of buffers to maintain this pH and prevent the formation of precipitates is not advised as they add further complications to an analysis that is already sensitive to contamination (Aminot *et al.*, 1997; Kérouel and Aminot, 1997). No attempt was made therefore to account for the salt effect in the measurement of ammonium since the majority of samples collected in this study were freshwater and of low salt concentration (Stewart and Elliott, 1996).

2.3.3 Ammonium (OPA method)

The ammonium concentrations were determined later from samples defrosted for TDN analysis. The samples were analysed for both determinands on the same day, but using a second ammonium procedure. Details of why ammonium concentrations were recorded in defrosted samples are given in §2.3.5. The fluorometric or orthophthaldialdehyde (OPA) method was preferred to the indophenol-blue technique as the blank was lower (0.5 µM) although the limit of detection was slightly higher (0.6 µM). The OPA technique has added advantages including a higher sensitivity, low salt effect and non-toxic reagents (Aminot *et al.*, 2001).

The determination of ammonium using the OPA method was carried out using a segmented flow analyser linked to a 40 cup autosampler (Kérouel and Aminot, 1997). The technique is based on a measurement of fluorescence that is produced from the reaction of ammonium with OPA. A working reagent was produced using the ratio 500:10:1:0.1 (ml) borate buffer (30 gl^{-1}): OPA in absolute ethanol (40 gl^{-1}) : sodium sulphite (8 gl^{-1}) : 30 % Brij solution (detergent)(Breviere, 2000). Although the working reagent was sensitive to light and the reaction required the addition of heat from a water bath (at 52 ^{o}C), the

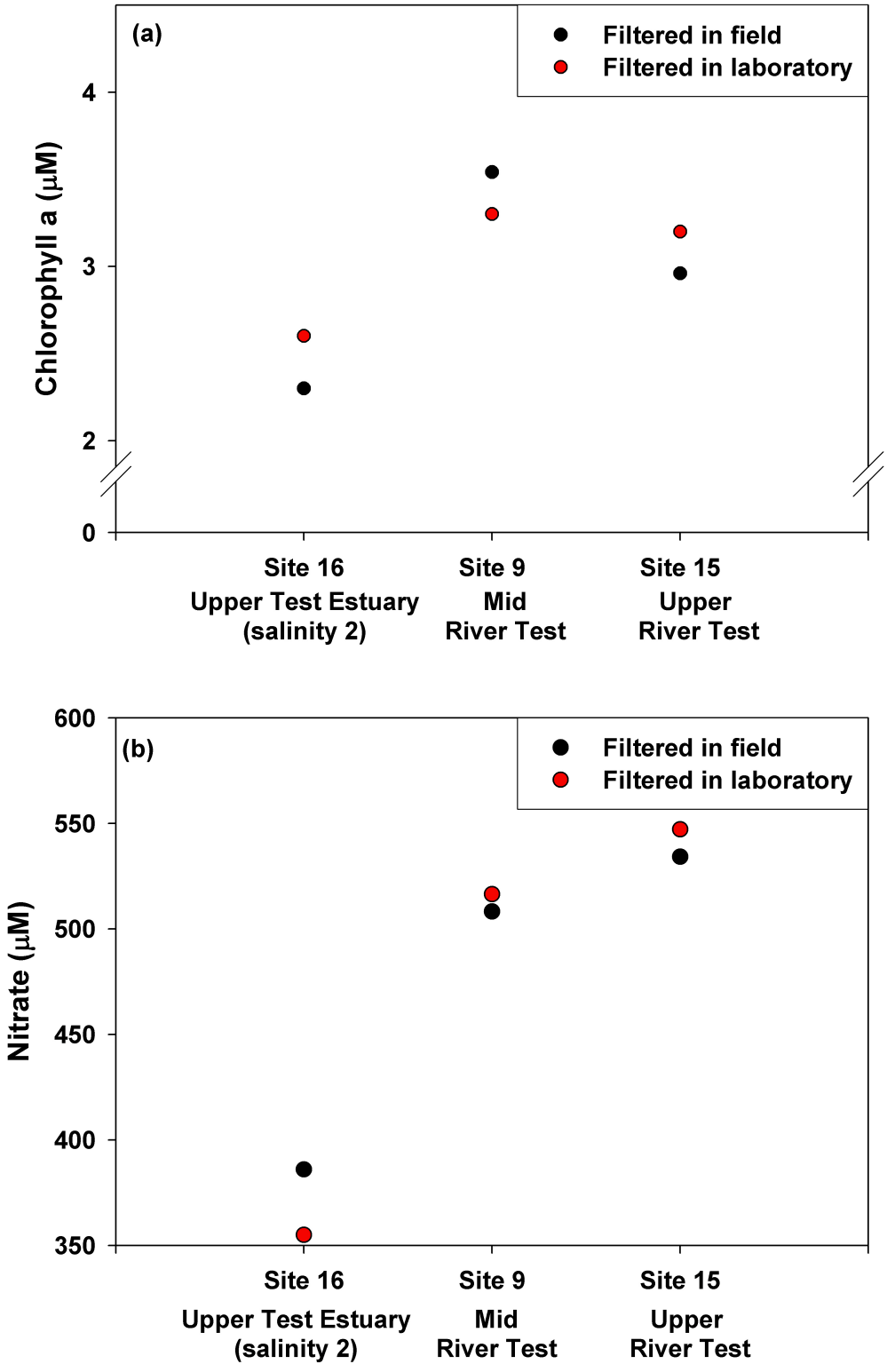

Figure 2.3. Experiment using (a) chlorophyll a and (b) nitrate samples collected in December 2001 to compare filtration in the field and laboratory. Where error bars are not shown, standard deviations (for up to 3 replicate measurements) are smaller than the size of the symbol. Note non zero intercept for y axis on panel (b).

analysis was far simpler and subject to less contamination than the indophenol blue method (Holmes *et al.*, 1999). Standards of ammonium chloride were prepared at 5, 10, 15 and 20 µM. Three sample cups were filled from each sample, therefore giving triplicate measurements, each of which had a replication error of < 0.3 µM.

Salt effect The salt effect in this technique was considered to be negligible (Aminot *et al.*, 2001). Despite this, samples were generally analysed using a 20 gl^{-1} NaCl carrier solution, although those with a salinity >20 were analysed using a carrier solution of 40 gl^{-1} NaCl.

2.3.4 Comparison of IPB and OPA ammonium analysis

Previous comparisons between the indophenol blue and OPA methods have shown a good agreement between the results (Kérouel and Aminot, 1997). Several samples of river water collected in March 2002 were analysed using both ammonium methods. There was a linear relationship between the two methods (slope = 1 and $R^2 = 0.86$), although slightly higher concentrations (0.47 µM) were obtained using the IPB technique (Figure 2.4). Possible reasons for this are discussed (§2.3.5). The OPA method had the greater precision, even though only three replicate measurements of each sample were made (*cf.* four in the indophenol blue method). It has also been reported that the indophenol blue method is less inaccurate at low concentrations (i.e. <0.5 µM) (Holmes *et al.*, 1999).

2.3.5 DOC and TDN

For DOC and TDN analysis, a Shimadzu 5000A total organic carbon (TOC) analyser was connected in series with an Antek 705E chemiluminescence nitrogen specific detector (Alvarez-Saldago and Miller, 1998). High purity oxygen was used as the carrier gas. 10 ml of sample was decanted into a pre-combusted glass vial (550 ^{o}C for 4 hours) and acidified with 50 µl of 10 % HCl to give a pH 2 – 3 and then purged externally with high purity nitrogen gas. These procedures of acidification and purging, remove dissolved inorganic carbon (DIC) as CO_2.

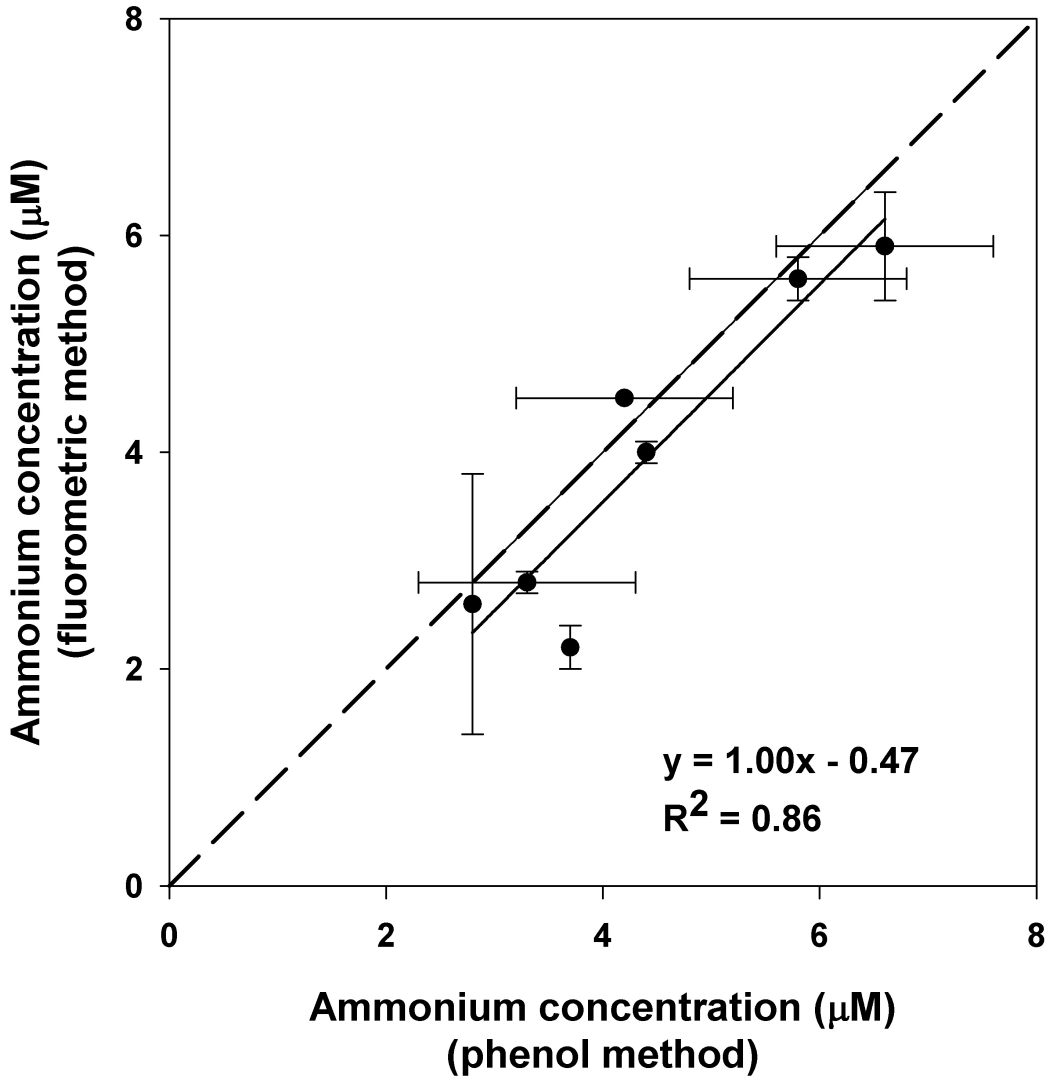

Figure 2.4. Comparison of ammonium concentrations measured using both the indophenol blue and OPA methods. Diagonal dashed line indicates a slope of 1. Solid diagonal line shows regression line fitted to data points. Error bars indicate standard deviation of usually four and three replicate measurements for the indophenol blue and OPA methods respectively.

There is some evidence to suggest that the purging may remove volatile organic carbon, although this is usually considered negligible (Spyres *et al.*, 2000). Although nitrogen is used for degassing the sample, it does not cause contamination as the analyser is unable to break N=N bonds (Stephen Duffin, Antek; Pers. Comm). The pre-filtration of water samples through GF/F filters removed particulate organic carbon (POC) thus only DOC is measured.

Prior to sample injection into the Shimadzu instrument the sampling syringe was washed four times with 100 µl of the degassed sample. Vaporisation of the sample occurred at 680 °C in the Al_2O_3 catalyst (impregnated with 0.5 % platinum) (Alvarez-Saldago and Miller, 1998; Cauwet, 1999). The combustion products were carried by high purity oxygen to the infra red gas analyser (IRGA) where the carbon dioxide (CO_2) content of the sample was detected. A fraction of the sample continued through to a second furnace at 900 °C and into the Antek total nitrogen analyser.

Nitric oxide (NO) within the sample was excited by ozone (O_3), producing nitrogen dioxide (NO_2) and, when NO returned to its original state, an electron was emitted which was detected by the chemiluminescence detector. The NO produced is proportional to the total nitrogen in the sample and other nitrogen products such as nitrogen gas (N_2) and NO_2 are assumed to be insignificant (Hansell, 1993). The instrument settings used are tabulated in Appendix E and a schematic diagram of the analytical system is presented (Figure 2.5). The peak areas were integrated using Shimadzu Class – VP software.

Estimation of DON precision Four injections were routinely taken from each TDN / DOC sample, although frequently one or more of these was rejected as an outlier. The Dixon test was used to identify potential outliers and these extreme values were closely examined to decide whether to reject or keep the data point (Farrant, 1997). The Dixon test ranks data values and calculates values from ratios (Equations 2.1 and 2.2) to compare with critical values at the 95 % significance level.

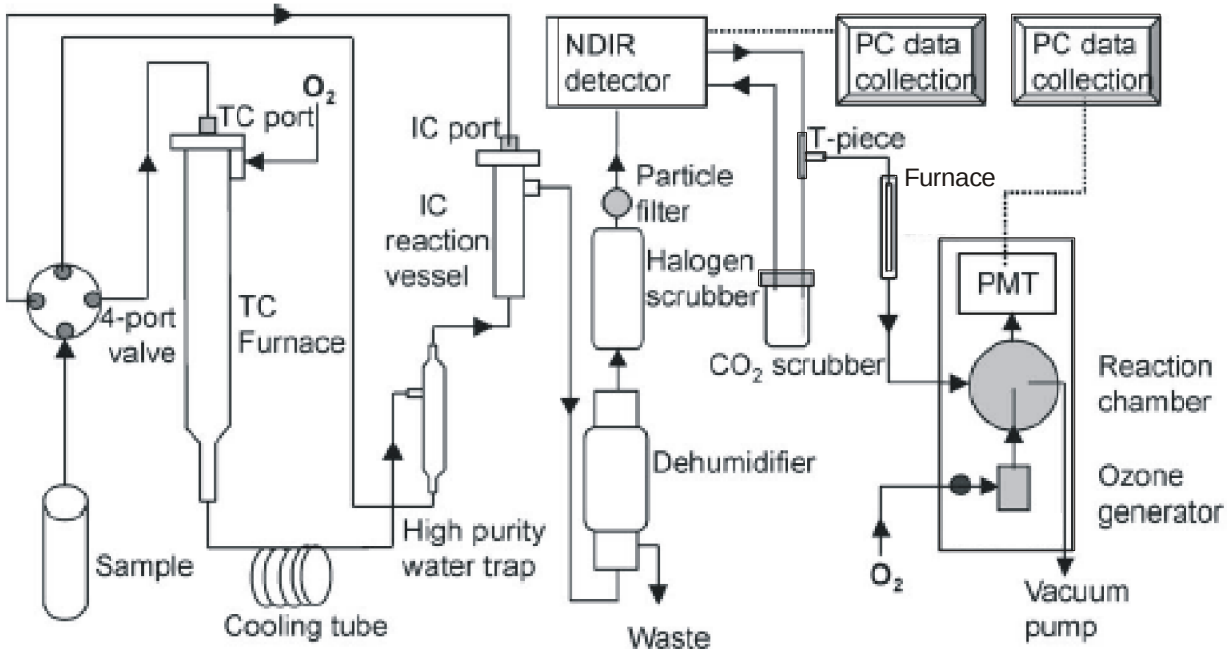

Figure 2.5. Shimadzu TOC 5000A in series with Antek 705E analytical system for determination of TOC and TDN. (Adapted from Badr *et al*, 2003.)

$$D_{\text{lowest}} = \frac{\chi_2 - \chi_1}{\chi_n - \chi_1} \quad \text{(Equation 2.1)}$$

$$D_{\text{highest}} = \frac{\chi_n - \chi_{n-1}}{\chi_n - \chi_1} \quad \text{(Equation 2.2)}$$

χ_1 = smallest data value

χ_2 = second smallest data value

χ_n = largest data value

There was no obvious pattern in the sequence of omitted replicates. In the literature the reported precision for DOC using the HTCO technique is 1 – 9 % coefficient of variation (Hedges *et al.*, 1993; Cauwet, 1999; Spyres *et al.*, 2000; Sharp *et al.*, 2002a), whereas TDN errors are <2 % (Alvarez-Saldago and Miller, 1998; Cauwet, 1999). The precision associated with the replicate measurements of samples carried out in this study was 7.1 % for DOC and 1.2 % for TDN, i.e. in good agreement with the literature. The replication error for the determination of DOC is known to be consistently higher than for TDN (Hansell, 1993). Using the definition that the limit of detection is three times the standard deviation of replicate blank measurements, the values for the DOC and TDN analysis were estimated to be 31 µM and 6 µM respectively (Frankovich and Jones, 1998).

DON concentration is determined indirectly using a difference method by subtracting the concentration of DIN (nitrate and ammonium) from TDN (Equation 2.3). The analytical error associated with DON is compounded from the standard deviation of four analyses of TDN, ammonium and three analyses of nitrate (Equation 2.4) (Hansell, 1993).

$$[\text{DON}]=[\text{TDN}]-[\text{DIN}] \quad \text{(Equation 2.3)}$$

$$\text{Propagated standard deviation} = \sqrt{(\Delta\text{TDN})^2 + (\Delta\text{DIN})^2} \quad \text{(Equation 2.4)}$$

Δ = standard deviation

In freshwater environments where the concentrations of dissolved inorganic nitrogen are several times higher than DON, the combined errors from the multiple analyses can result in high errors for DON and therefore give DON concentrations of zero or that are negative (Hansell, 1993; Hopkinson *et al.*, 1993; Hedges *et al.*, 1994a; Bronk *et al.*, 2000). The mean coefficient of variation in the determination of DON from this study was 22 %,

which was within the range (between 10 and 27 %) reported in the literature (Hedges *et al.*, 1993; Hedges *et al.*, 1994a; Servais *et al.*, 1999; Sharp *et al.*, 2002b).

When negative values were determined for DON in this study, the strategy was to repeat analysis of TDN and nitrate to further constrain the errors. Negative DON concentrations may be attributed to the loss of volatile organic nitrogen during the purging stage of the TDN analysis as this has been found to cause up to 5 % losses in DOC concentrations (Statham and Williams, 1999). The flocculation of humic matter has been observed in some samples post freezing, which may account for lower TDN concentrations than nitrate and result in negative values for DON (Tranvik, 1990). Typically in river and estuarine water, analysis of DOC and TDN does not require sample dilution. As the measurement of nitrate requires a ten fold dilution this probably contributed to the largest propagated error in DON.

Storage stability Samples for TDN and DOC determination are generally stored using freezing or adding hydrochloric or phosphoric acid at room temperature or 4 ^{o}C (Tupas *et al.*, 1994; Spyres *et al.*, 2000). Mercuric chloride is avoided as this can poison the catalyst (Kaplan, 1994). In this study samples were frozen immediately after filtration and stored at –20 ^{o}C in precombusted glass bottles. Although glass is the preferred storage container to minimise contamination, it becomes brittle due to the combustion process and therefore there is a high risk of breakage, even when sufficient room is left for expansion (Tupas *et al.*, 1994; Holmes *et al.*, 1999). This was a problem in this study and resulted in some loss of samples.

Freezing is considered an acceptable method for storing TDN samples (Cornell *et al.*, 1995; Cape *et al.*, 2001), although little attention has been given to how freezing affects the nitrogen species within the TDN sample. In particular, the nutrients of lowest concentrations are most susceptible to change (Morse *et al.*, 1982; Chapman and Mostert, 1990; Dore *et al.*, 1996). The ideal storage method would be to store all nitrogen species in the same way prior to analysis (Dore *et al.*, 1996), but freezing is not a recognised method for preserving ammonium.

Ammonium was measured in the frozen TDN samples and compared with concentrations measured from bottles fixed with phenol post-filtration. Samples collected from July 2001 to February 2002 measured using the indophenol method had higher concentrations of

ammonium after freezing (Figure 2.6a). From March 2002, samples that were collected and frozen for DOC and TDN determination were analysed for ammonium using the OPA technique immediately on defrosting. Figure 2.6b shows a comparison between ammonium preserved in the field and analysed with the IPB method, with concentrations measured using the OPA method in defrosted samples. Concentrations in defrosted samples were similar to those preserved in the field. Although the analyses prior to March 2002 using the IPB method suggested an increase in ammonium concentrations on freezing, this was not detectable using the OPA method.

Ammonium is volatile and is often reported to change with inappropriate storage (Aminot and Kérouel, 1995). However, ammonium concentrations are often reported to decrease on storage rather than increase as observed in this study. A reason for decreasing ammonium may be the microbial transformation of ammonium to DON (Cape *et al.*, 2001). This change in nitrogen form would not be observed in the overall measurement of TDN. The increase in ammonium observed in this study may depend on interference in the indophenol blue technique from other nitrogen containing compounds. For example Cape *et al.* (2001) found a colorimetric response from free amino acids (glycine and methionine) in rain water, leading to an overestimation of the DIN within the TDN and therefore an underestimation of the DON.

The freezing process may have caused larger organic nitrogen compounds to break down producing a higher abundance of compounds that were detectable using the indophenol blue method. The OPA method is more specific to ammonium compounds and therefore had no interference. In response to these experiments, all DON concentrations were calculated using measurements of ammonium from defrosted TDN samples to ensure an accurate determination of DON by verifying concentrations of DIN immediately prior to TDN analysis.

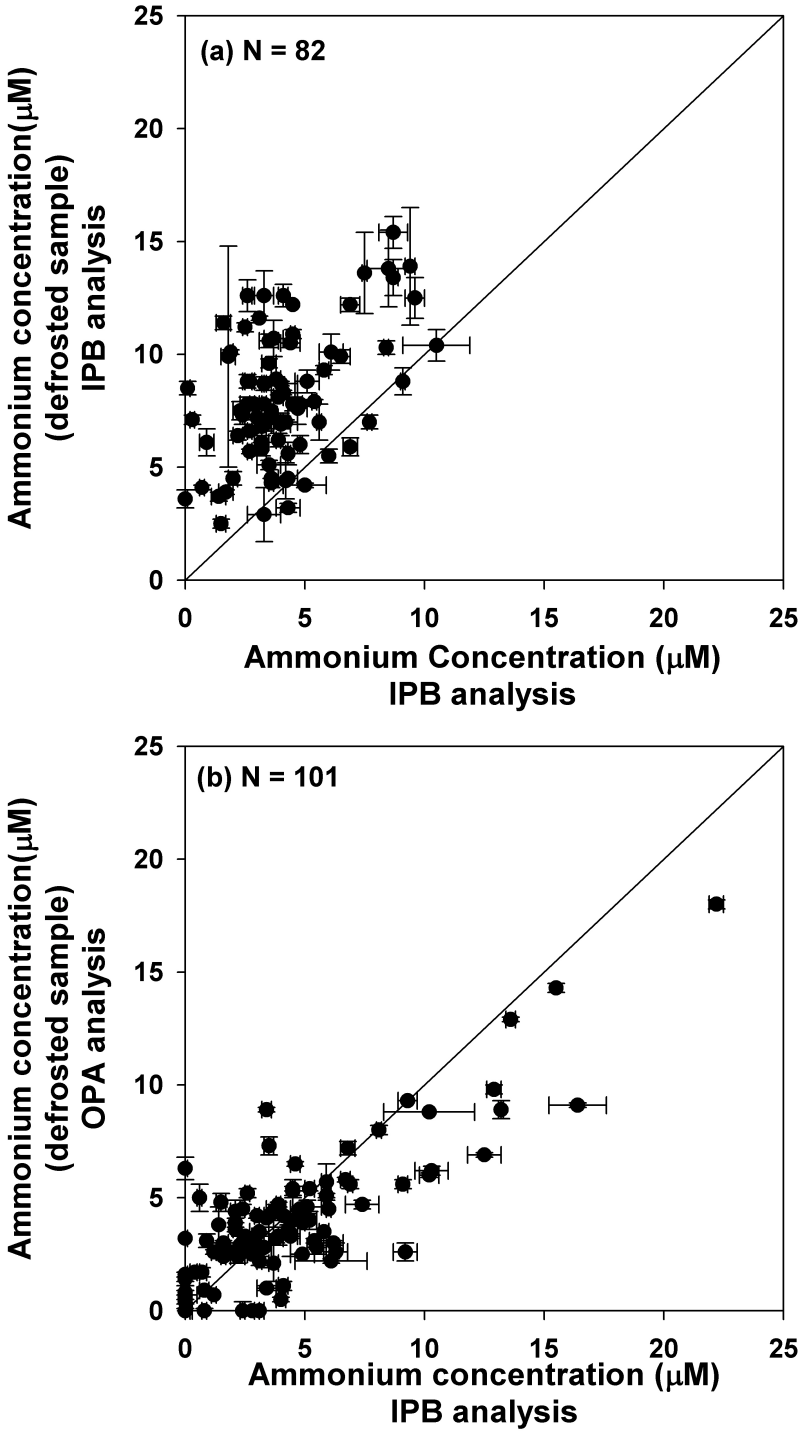

Figure 2.6. Comparison of ammonium concentrations between samples stored in the field with phenol and determined using indo-phenol blue method relative to defrosted samples and ammonium determined using (a) the indo-phenol blue method (July 01 to February 2002) and (b) the OPA method (March 2001 to December 2002). N = number of samples.

Salt effect There appears to be a negligible salt effect using the HTCO method for determination of DOC and TDN (Perdue *et al.*, 1993). This technique does necessitate sample dilution when a large number of high salinity samples are being processed as this minimises the build up of NaCl on the Pt-coated catalyst (Cauwet, 1994). The halogen scrubber should retain the chlorine, but if this becomes clogged, the chlorine will destroy the IRGA (Cauwet, 1999). Since the majority of samples in this study were freshwater, this dilution was not required, thereby removing an additional source of error in the determination of DON.

DOC blanks

Accurate measuring of blanks is considered problematic in the determination of DOC (Hall *et al.*, 1992). There are three types of blank, 1) the water blank, which cannot be completely removed due to the lack of carbon free water (Williams *et al.*, 1993; Spyres *et al.*, 2000), 2) the reagent or acid blank, and 3) the system blank (Benner and Strom, 1993). The water and reagent blanks are closely linked as obtaining low carbon water will reduce the reagent blank value. Low carbon water was obtained by UV irradiation (for 4 hours) of fresh Milli-Q water, which was used within 24 hours (Williams *et al.*, 1993; Buesseler *et al.*, 1996). The acid was prepared in clean glassware and diluted with UV irradiated Milli-Q water. All glasswear was cleaned using either washing with 10 % hydrogen peroxide or by combustion at 550 ^{o}C for 4 hours.

Although all standards and samples were acidified, therefore acquiring a reagent/acid blank, the largest and most difficult to quantify is the system blank. It is thought that the main cause of the system blank is the catalyst, especially when it is made of platinum coated quartz wool as it can exhibit a carry over effect from samples with a high carbon content (Benner and Hedges, 1993; Miller *et al.*, 1993). The HTCO instrument used quartz beads, which have a lower capacity to absorb carbon dioxide (CO_2) (Cauwet, 1999). Care was taken to obtain consistently low instrument blanks following the installation of a new catalyst, as thorough washing with blank water was required prior to sample analysis. (Cauwet, 1994). The Shimadzu 5000A has an ultra pure water trap that can be used to assess the system blank. Concentrations of DOC from this trap were often lower than the mean combined water and system DOC blank of 58 µM ± 49. When outliers were omitted, the majority of samples were analysed with a blank of 25 µM ± 11. This was

closer to the range of 10 – 33 µM carbon reported (Benner and Hedges, 1993; Hansell, 1993; Buesseler *et al.*, 1996; Bahr *et al.*, 2003). The nitrogen blank associated with the HTCO analysis was considerably lower as the combined nitrogen instrument and water blank was 6 µM. This was similar to the range of blanks reported in the literature (Hansell, 1993; Bahr *et al.*, 2003)

Potential sources of contamination to the blank and samples were the sample preparation using acidification and purging. A series of experiments was carried out to investigate whether these factors influenced the concentrations measured. Firstly the acidification of the sample was carried out with 10 % hydrochloric acid (HCl) prepared from UV irradiated Milli-Q water and comparisons were made with 10 % phosphoric acid. There was no apparent change to the efficiency of the acidification, although hydrochloric was preferred as it is less corrosive to the platinum coated catalyst.

Comparisons were made between degassing using nitrogen and helium, both of which were high purity inert gases. The helium showed no marked change in the peak areas from the nitrogen and therefore it was concluded that nitrogen was an acceptable purging gas. A minimum purging time of 5 minutes was used for each sample, although the length of purging time was usually 15 minutes.

Oxidation efficiency

Until the chemical composition of DON is better understood it is very difficult to select standard compounds that represent the lability of the DON in seawater and freshwater samples (Sanders and Jickells, 2000; Cape *et al.*, 2001). Although nitrate is the ideal choice of standard as it is a major component of river water, it cannot represent the complex organic molecules in DON (Walsh, 1989). To consider the oxidation efficiency of the technique, it was necessary to compare a range of compounds at a variety of concentrations (Spyres *et al.*, 2000).

Solutions of urea (0.303 gl^{-1}), caffeine (0.4855 gl^{-1}) and potassium nitrate (1.01 gl^{-1}) were freshly prepared from dried chemicals and diluted with UV irradiated Milli-Q water to 20, 200, 500 and 700 µM. These were measured using HTCO and the chemiluminescent nitrogen specific detector and the peak areas were compared. Urea was considered first as it was a compound known to be present in natural waters (Frankovich and Jones, 1998).

Low oxidation efficiencies were observed, particularly at high concentrations (Cauwet, 1999), which posed a problem since the majority of samples from the study area were expected to lie within in the upper quartile of the calibration range. Caffeine was selected as a suitable standard for the DOC and TDN analysis as the oxidation efficiency relative to potassium nitrate was comparable to the literature (Table 2.3).

Table 2.3. Summary of literature oxidation efficiency (percentage recovery) for caffeine and urea using HTCO techniques. NA indicates data not available

	Oxidation efficiency (%)	
	Urea	**Caffeine**
This study	91	97
Walsh, 1989	100.2	NA
Fry *et al.*, 1993	NA	103
Koike and Tupas, 1993	100	102
Fry *et al.*, 1996	NA	98
Qian and Mopper, 1996	NA	102.7
Alvarez-Saldago and Miller, 1998	101	97
Frankovich and Jones, 1998	94.3	NA
Kahler and Koeve, 2001	97	NA
Bahr *et al.*, 2003	91.2	79.5

Typical caffeine calibrations for both carbon and nitrogen are presented in Figure 2.7. Since oxidation efficiencies >100 % were achieved comparing caffeine and potassium nitrate standards of the same concentration, no attempt was made to correct for the oxidation efficiency (Hopkinson *et al.*, 1993). Carbon rich impurities in the dried standard may account for oxidation efficiencies of over 100 % (Qian and Mopper, 1996).

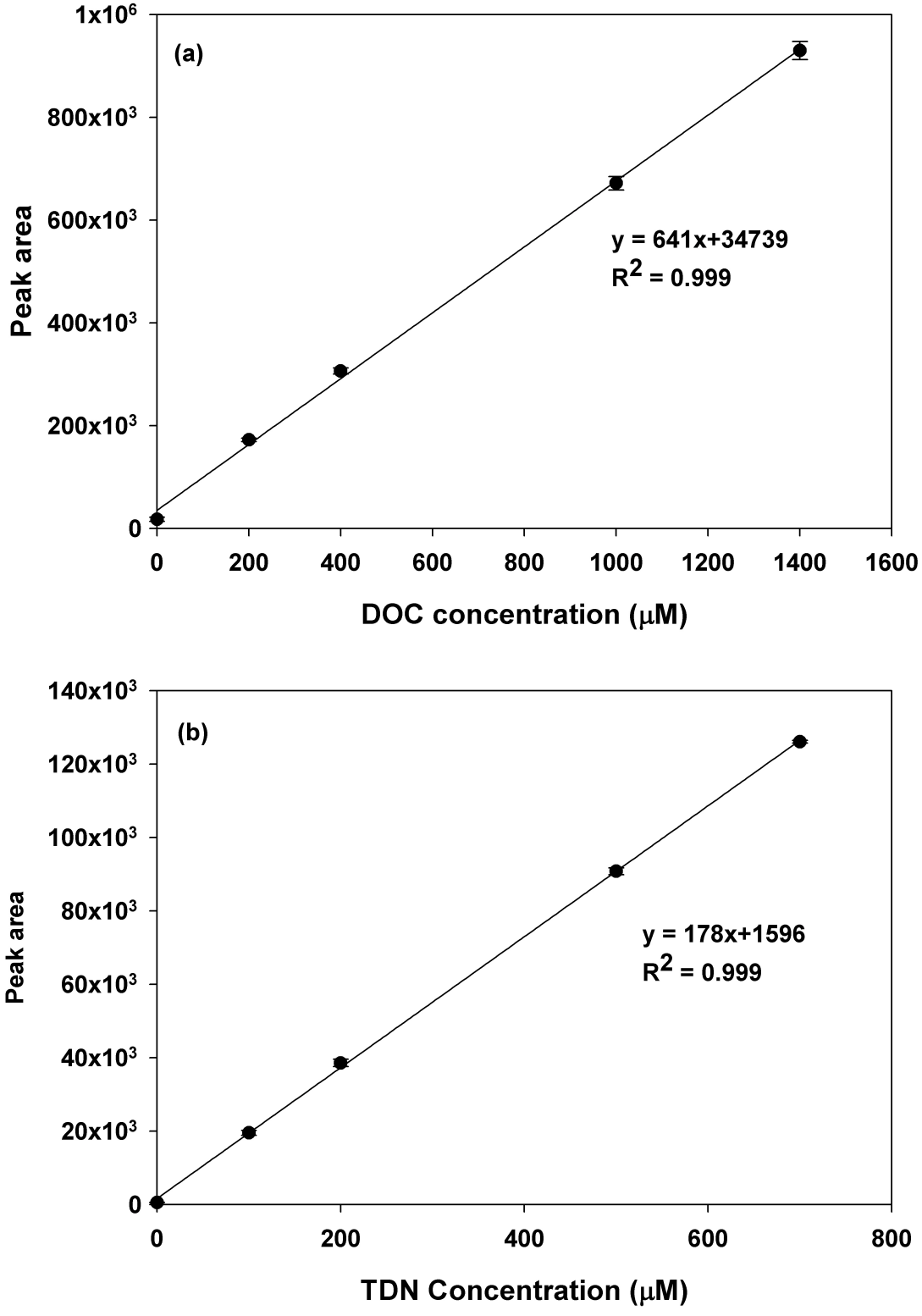

Figure 2.7.Typical caffeine calibration curve for (a) DOC and (b) TDN peak areas. (Data collected on 22nd April 2004).

Calibration consistency

At the beginning of each daily batch of analyses a 4 point calibration curve was obtained with caffeine standards at nitrogen concentrations of 100, 200, 500 and 700 µM (containing carbon at 200, 400, 1000 and 1400 µM). This range was selected to bracket the concentrations of TDN found in both fresh and estuarine water samples. The peak areas from four injections of each standard were subjected to the Dixon outlier test to remove any anomalous points (Miller and Miller, 1993) (Equation 2.1 and 2.2). The peak area of the blank water was subtracted from the mean peak area prior to plotting the calibration curve. The same blank (water plus instrument blank) was subtracted from the samples. Ideally only the instrument blank should be subtracted but this cannot be determined separately until a totally carbon free source of water has been obtained (Cauwet, 1994). The order in which the standards were analysed at the beginning of the daily batch and in between sets of samples is shown in Appendix F. There was some day-to-day variation in the slope of the calibration curve (see also Walsh, 1989). Consequently, although calibrations were consistently linear, it was necessary to carry out a full calibration prior to each batch of analyses and between sets of 5 – 8 samples a blank and both an inorganic (potassium nitrate) and organic (caffeine) standard were analysed to check for instrument drift.

Certified reference material

Certified reference material (CRM) was available from the University of Miami in the form of deep Sargasso seawater (21 µM N and 44 – 45 µM C) and low carbon (negligible N and 2 µM C). Although these acidified (phosphoric acid) and highly stable standards are used widely by HTCO analysts, it has been acknowledged that they are unlikely to contain labile DON and DOC that are present in surface water samples (Hopkinson *et al.*, 1993). Typically, good agreement was seen between the nitrogen measurements and concentrations in the deep Sargasso Sea CRM (21 µM ±5). However, the values for DOC were lower than expected with 41 µM ±18 being the mean concentration. The large error associated with the carbon CRM may be due to no standards < 200 µM C being analysed to calibrate the HTCO.

GANE community TDN intercalibration

A variety of different analytical techniques were used to determine DON in samples collected during the GANE project therefore a community wide intercalibration exercise was carried out to compare the various TDN analytical methods used. Seven laboratories participated with some laboratories using more than one analytical technique. The four analytical techniques compared were persulfate oxidation, HTCO, ultraviolet (UV) oxidation and Kjeldahl digestion. The analysis of standards supplied by the GANE intercalibration exercise was not completed as part of this project in sufficient time to be included in the GANE report (Evans *et al.*, 2002), but a comparison can be drawn between the conclusions found in the report and the subsequent analysis.

Each of the laboratories was supplied with a series of 20 ml ampoules containing either a standard solution prepared from thymine, EDTA, glycine or urea or alternatively a marine or river water sample. These standards were prepared using inorganic nitrogen free water within a low (0 – 20 µM) or medium (100 – 200 µM) concentration range. A third concentration range of > 3000 µM was also prepared but many analysts declined to analyse these as they were greater than the analytical range of the majority of natural water samples. The results from the high concentration standards will not be discussed further. The pre-combusted (550 ^{o}C for 5 hours) glass ampoules were sealed and frozen at –19 ^{o}C and delivered to participating laboratories in dry ice (Evans *et al.*, 2002).

Conclusions from the cross method comparisons suggest that there was a good degree of agreement between low concentration standards measured in laboratories 1, 2 and 3, even though they use different analytical techniques (Figure 2.8). The groups as a whole were better able to analyse the medium than low standards, although laboratory 2 consistently achieved a low oxidation recovery with the medium concentration standards (Evans *et al.*, 2002). The red bar indicates the concentrations of TDN determined in this study. The concentrations were in good agreement in the medium concentration standards and slightly higher than actual standards for the low concentration standards.

Each laboratory was also provided with two ampoules each of marine and freshwater samples. These were analysed in an identical manner and the group mean was calculated and plotted as a horizontal line on Figure 2.9. Again the concentrations measured by laboratories 1, 2, and 3 were in closest agreement for the riverine samples.

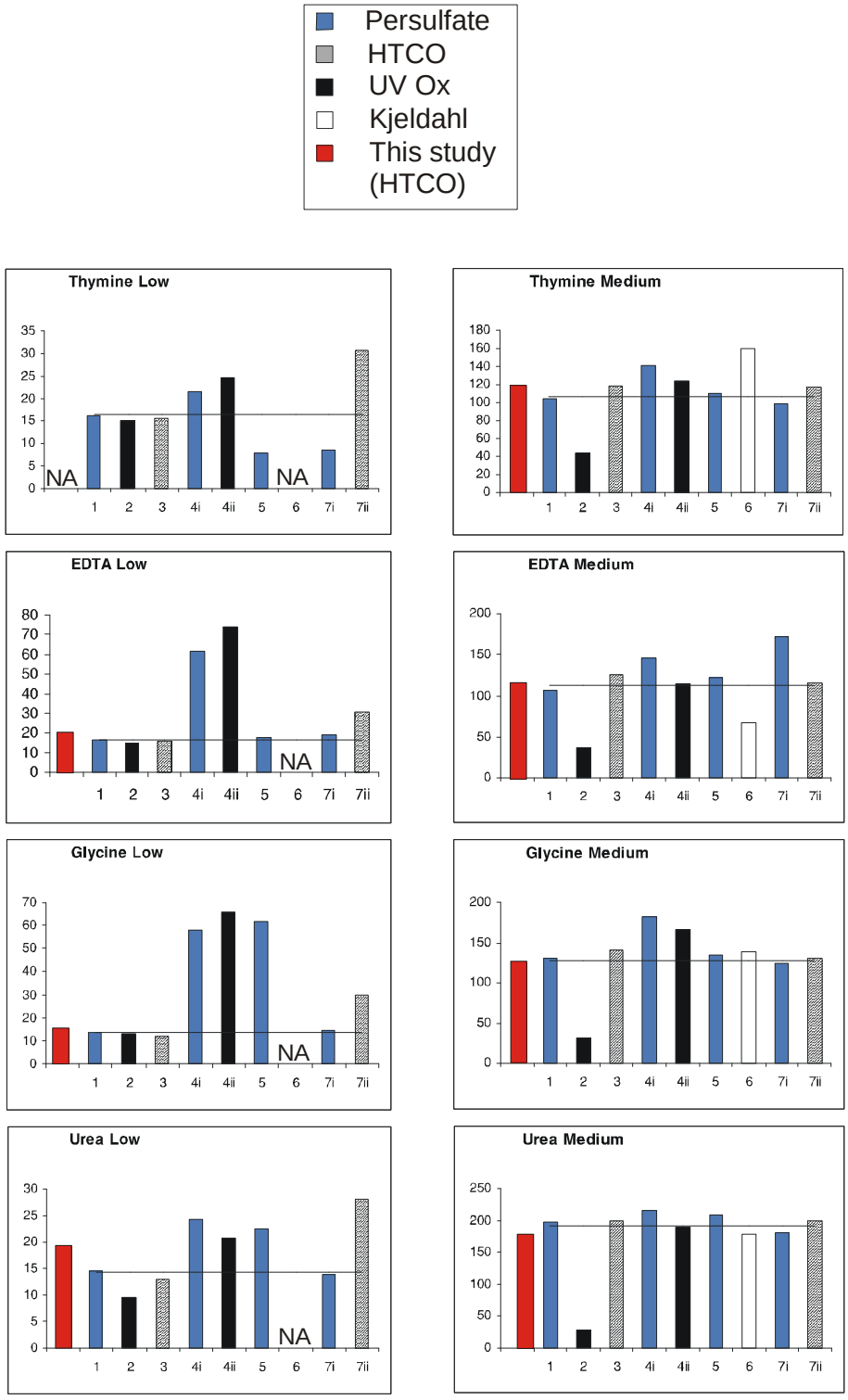

Figure 2.8. DON standards from the GANE DON intercalibration exercise 2002. The key indicates the various analytical techniques used in the exercise. Red bars indicate analyses performed using the HTCO technique in this study. Each analytical laboratory was labelled 1 to 7 and those laboratories that conducted more than one technique were labelled i and ii as appropriate. The concentration of the distributed standard is represented by the black horizontal line. NA indicates not analysed. Adapted from (Evans *et al.*, 2002).

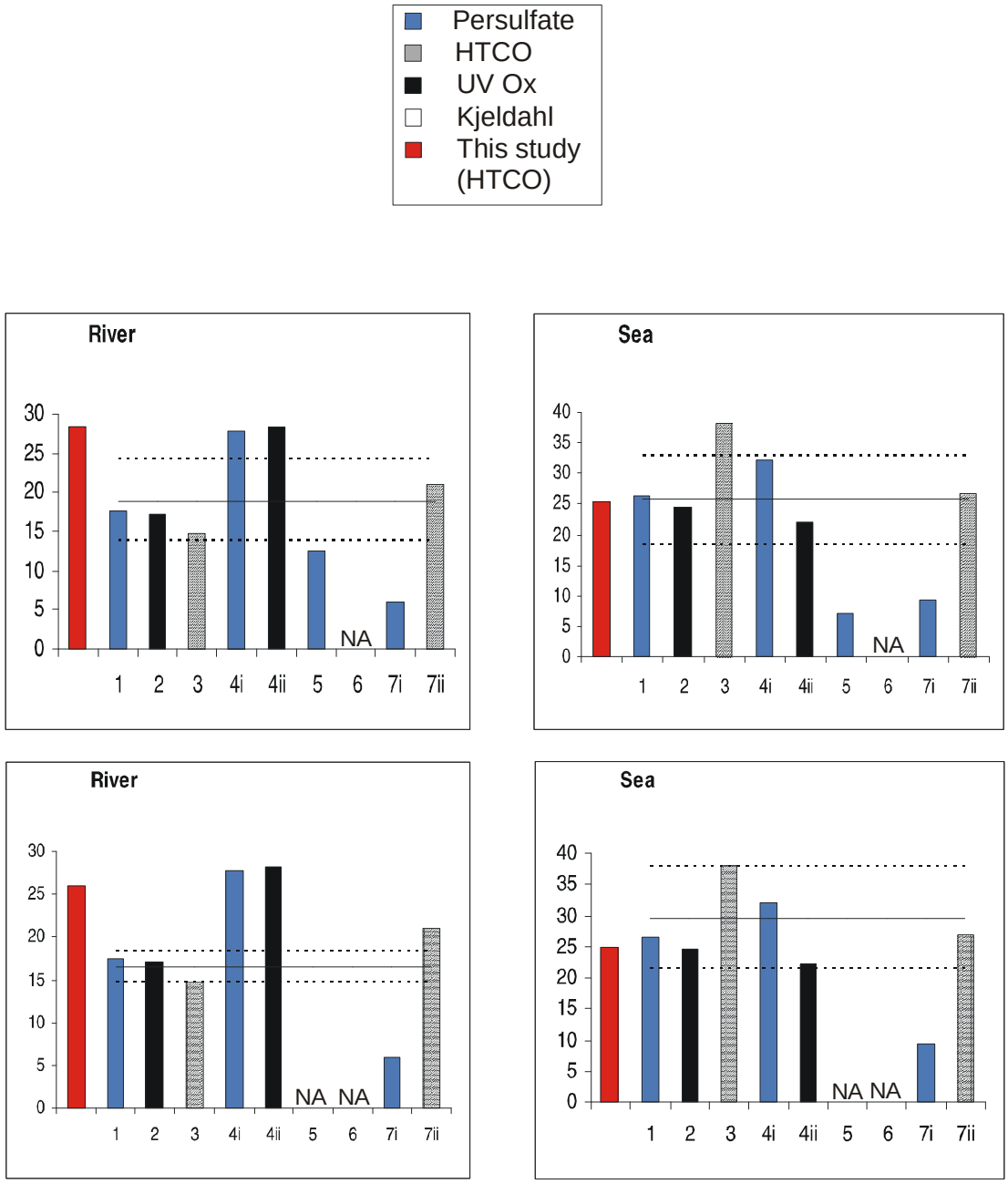

Figure 2.9. River and marine samples from GANE DON intercalibration exercise 2002. The key indicates the various analytical techniques used in the exercise. Red bars indicate analyses performed using the HTCO technique in this study. Each analytical laboratory was labelled 1 to 7 and those laboratories that conducted more than one technique were labelled i and ii as appropriate. The black horizontal line is the mean group concentration of the sample and the dashed horizontal lines indicate the 95% confidence limits for the group mean. Adapted from (Evans *et al.*, 2002). NA indicates not analysed.

However, overall laboratories 1, 2, and 4ii were the only groups to measure all samples within the 95 % confidence limits (Evans *et al.*, 2002). Poor oxidation efficiencies were observed for groups 5 and 7 and group 7 had a great discrepancy between the two analytical methods. The red bars indicate the results from this study. There was good agreement, within the 95 % confidence intervals for the marine samples. However, the river samples were consistently overestimated; this was similar to the low concentration standards. The poor accuracy of the HTCO method at low concentrations is not of great concern since TDN in the River Test and estuary is ten fold higher than these low standards. More importantly the HTCO is able to measure the mid-range standards which are a better approximation of the TDN measured in samples collected during this study. The overall conclusion of the intercalibration exercise was that within the GANE community there was an overestimation of DON concentrations and slightly less so at medium than at low concentrations. The exception was the UV oxidation method which appeared to underestimate clearly at medium concentrations.

2.3.6 PON and POC

Analysis of the particulate organic nitrogen (PON) and carbon (POC) from the GF/F filters was carried out using a Carlo Erba EA1108 CHNS-O elemental analyser. The particulate nitrogen retained by the filter was expected to be mainly organic, as inorganic nitrogen is removed in the filtrate. The filters were oven dried at ~ 60 ^{o}C for 24 hours. A filter punch was used to cut two 17.5 mm diameter circles from each 47 mm GF/F filter, which were individually folded and compacted within tin capsules. Prior to analysis the tin capsules were stored at room temperature in a desiccator with silica gel.

The elemental analyser was calibrated using blank filters and sulphanilamide standard which contains 41.85 % carbon and 16.27 % nitrogen. The analysis uses oxygen and helium as carrier gases and involves flash combustion followed by a reduction of the nitrogen oxides to nitrogen gas (N_2) and carbon dioxide (CO_2) for detection using thermal conductivity (Verardo *et al.*, 1990). The concentration of nitrogen and carbon was calculated per litre of water filtered, assuming a uniform distribution of particulates over the filter. The analysis had a blank of 0.9 µM and a limit of detection of 0.06 µM for both POC and PON. The precision of the analytical technique analysing two replicates from each GF/F filter was 5 % for POC and 9 % for PON.

2.3.7 Chlorophyll a

Fluorometric determination of chlorophyll a was carried out using a Turner design 10 – AU fluorometer (Parsons *et al.*, 1984). The fluorometer was regularly calibrated using a 1:10 dilution of 1000 $\mu g l^{-1}$ chlorophyll a standard (Sigma Chemicals), stored at –20 ^{o}C. The amount of pigment in the standard was determined photometrically using Equation 2.5.

$$\text{Chlorophyll a } (\mu g\ l^{-1}) = 11.85\ E_{664} - 1.54\ E_{647} - 0.08\ E_{630} \quad \text{(Equation 2.5)}$$

$$\text{Chlorophyll a } (\mu g\ l^{-1}) = P \times \frac{v}{V} \quad \text{(Equation 2.6)}$$

E = absorbance at a specific wavelength (corrected by the reading at 750 nm)

P = fluorometer reading

v = volume of acetone (ml)

V = volume of water filtered (ml)

Each sample was filtered through a Whatman 25 mm (diameter) GF/F filter using an in-line swinex filtration system. The filters were stored frozen and brought to room temperature in the dark prior to analysis. Each filter was placed in the bottom of a centrifuge tube with 8 ml acetone (90 %) and sonicated for 30 seconds. The tubes were then centrifuged for 10 minutes at 3000 rpm and the supernatant analysed using the fluorometer. Equation 2.6 was used to calculate the chlorophyll a concentration (Parsons *et al.*, 1984). In December 2000 and January 2001, 3 replicate filters were collected for analysis from sites in the upper, mid and lower reaches of the study area (sites 16, 9 and 15). The mean standard deviation for three replicate measurements was 4.8 % and the limit of detection was 0.7 $\mu g\ l^{-1}$.

2.4 ENVIRONMENT AGENCY SAMPLING AND ANALYSIS PROCEDURES

The monthly nutrient samples collected by the Environment Agency (EA) at a number of sites on the River Test and estuary, are analysed in regional laboratories for ammonium and nitrate. The EA does not analyse for particulate organic nitrogen or DON. According to Tony Lee of the Environment Agency, Southern Region (pers. com:), nitrate

concentrations are determined indirectly by subtracting nitrite concentrations from TON. The TON determination is similar to that discussed above for nitrate, although copper / hydrozene is used as the reducing agent rather than a cadmium column (HMSO, 1981b).

The analysis of samples for ammonium concentration is conducted using different methods for freshwater and saline samples. The freshwater ammonium measurements are determined spectrophotometrically using hypochlorite and salicylate reagent in the presence of sodium nitroprusside. In contrast the estuarine samples are analysed using hypochlorite and phenol in the presence of potassium ferrocyanide (HMSO, 1981a).

3 CHAPTER THREE. TEMPORAL VARIATIONS IN THE INORGANIC AND ORGANIC NITROGEN IN THE RIVER TEST AND ITS ESTUARY

3.1 INTRODUCTION

Temporal changes in river and estuarine nutrient concentrations are influenced by seasonality in rates of primary production and nutrient consumption, as well as external factors such as changes in river flow. Nutrient inputs from diffuse sources (for example agricultural runoff of fertilisers and atmospheric deposition) may also have an impact but are more difficult to quantify. In contrast, point source effluents from sewage treatment works (STW) and fish farms can be more readily monitored.

A monthly sampling survey commencing in July 2001 was carried out along a 17 km section of the lower reaches of the River Test and upper estuary to investigate the temporal variation in nutrient concentrations over an 18 month period. Different processes were expected to influence the saline and freshwater systems therefore data are presented separately to investigate temporal nutrient variation. The fractions of total nitrogen and carbon within these systems were calculated to characterise the proportion of nitrogen (and carbon) represented by dissolved organic matter (DOM). This study represents the first estimates of the total nitrogen budget for the River Test and upper Test estuary, as routine nutrient monitoring by the Environment Agency does not include determination of dissolved organic nitrogen (DON).

Although there is no current legislative limit to nutrient concentrations in UK estuaries, international agreements (European Community Directives, The OSPAR convention and the North Sea Conference Declarations) require the reduction of nutrients from land-based sources entering the sea (Nedwell *et al.*, 2002). The calculation of inorganic nitrogen loads from many UK rivers by Nedwell *et al.* (2002) has permitted a comparison of estuaries and their nutrient status. In this chapter the combination of instantaneous river flow from just above the tidal limit with nutrient concentrations (organic and inorganic) was used to determine the nitrogen load from the River Test into the estuary. A better understanding of seasonal changes in river and estuarine DON in relation to other nutrients will clarify the DOM sources and sinks as well as providing a more complete nitrogen budget for this system and total riverine nitrogen loading of the Test estuary.

3.2 METHODOLOGY

Water samples were collected from River Test and estuary between Mottisfont and Redbridge railway station over 18 months between July 2001 and December 2002 (Figure 2.1). The aim of sampling on a monthly basis was to characterise temporal variations in nutrient concentrations. The samples were analysed using a standard suite of analytical procedures detailed in §2.3.

3.3 RESULTS

3.3.1 Field measurements

In situ temperature and conductivity measurements were taken using a WTW salinometer from surface waters at each sampling site, with the exception of sites 1 and 18, where measurements were also taken at depth (Table 2.1). The data from these measurements are presented on a monthly basis in Appendices B and C.

Figure 3.1a presents the temperature measurements at every sampling site throughout the course of the 18 months. The range of temperatures measured was from 4.6 to 19.6 ^{o}C with the highest temperatures observed in saline rather than freshwater samples. Predictable seasonal changes in water temperature were measured, slightly warmer water temperatures were measured in winter 2002 than 2001.

Salinity was measured in water samples on return to the laboratory and was only detected in samples collected from the upper estuary and lower reaches of the River Test (Appendix D). Sites 18, 17, 1, 2, 6 and 6a (Table 2.1) were consistently saline or had regular saline intrusion, the extent of saline intrusion being dependant upon the spring-neap tidal cycle. The time of sampling was adjusted to obtain water samples from the period of maximum ingress of water. In June 2002 a maximum salinity of 33.1 was measured at site 1.

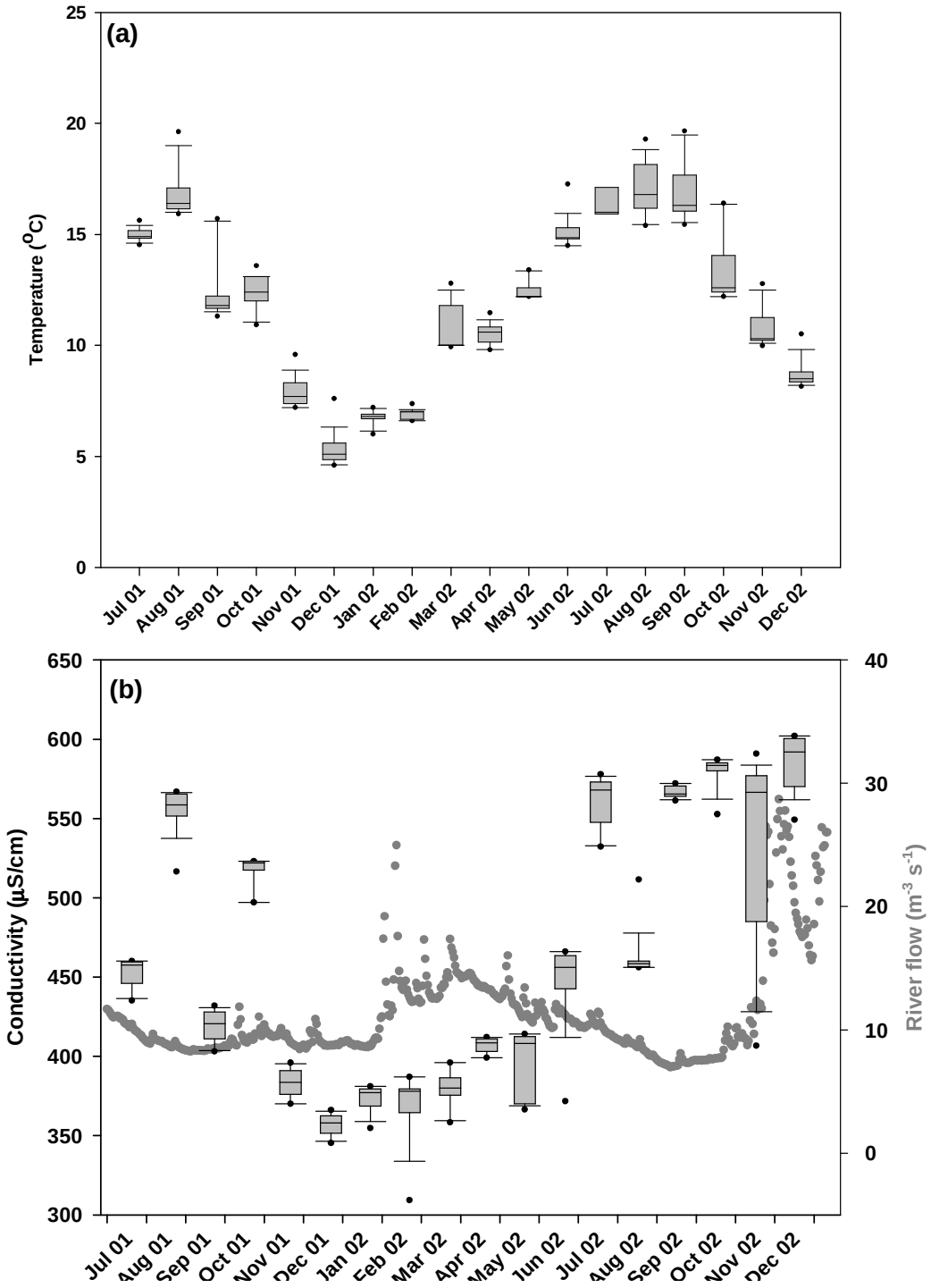

Figure 3.1. (a) Water temperature and (b) freshwater conductivity (EC) measurements from the River Test and estuary represented using box and whisker plots. The box upper boundary represents the 75th percentile and 25th is that closest to zero. The whiskers above and below the box indicate the 10th and 90th percentiles. The line within the box represents the median and outliers are represented by dots. Computations of whisker percentiles require more than 5 data points. Daily mean river flow is shown in grey.

In freshwater samples water conductivity (EC) was determined which is a measure of the total dissolved ions in the water. The EC in freshwater samples was between 300 and 650 μS cm^{-1} (Figure 3.1b). Highest measurements were recorded in summer of 2001 and 2002, with the lowest EC from winter 2001 to spring 2002. Although there was no apparent relationship between water conductivity and flow, the EC remained high following summer 2002 and did not decline in November and December as seen in 2001. These high water conductivities coincided with the highest flows measured during the 18 month survey.

3.3.2 Nutrient observations

Nutrient data at all sites Nutrient concentrations from all sites (including those from parallel stretches of the river) gave a good indication of the range of concentrations observed in the river to estuary continuum. These measurements of nitrate, ammonium and chlorophyll a from July 2001 – December 2002 are summarised in Figure 3.2.

A wide range of nitrate concentrations was measured (18 to 568 μM; Figure 3.2a), with the majority of concentrations being above 400 μM. The lowest concentrations were measured in saline samples and clear temporal variations were apparent. Low concentrations were measured in summer 2001 and these increased during the autumn to reach peak nitrate concentrations in February 2002. A spring to summer decline in nitrate was followed by autumnal increases in both 2001 and 2002.

Ammonium concentrations measured during the 18 month survey were generally less than 10 μM. A number of saline samples contained higher concentrations and did not follow the same temporal variation as the freshwater samples (Figure 3.2b). This was particularly pronounced in the latter half of 2002 when there was regular sampling of estuarine sites 17 and 18, downstream of Redbridge. The data showed some degree of temporal variation in ammonium concentrations, with decreasing concentrations at the majority of sites between July and October 2001.

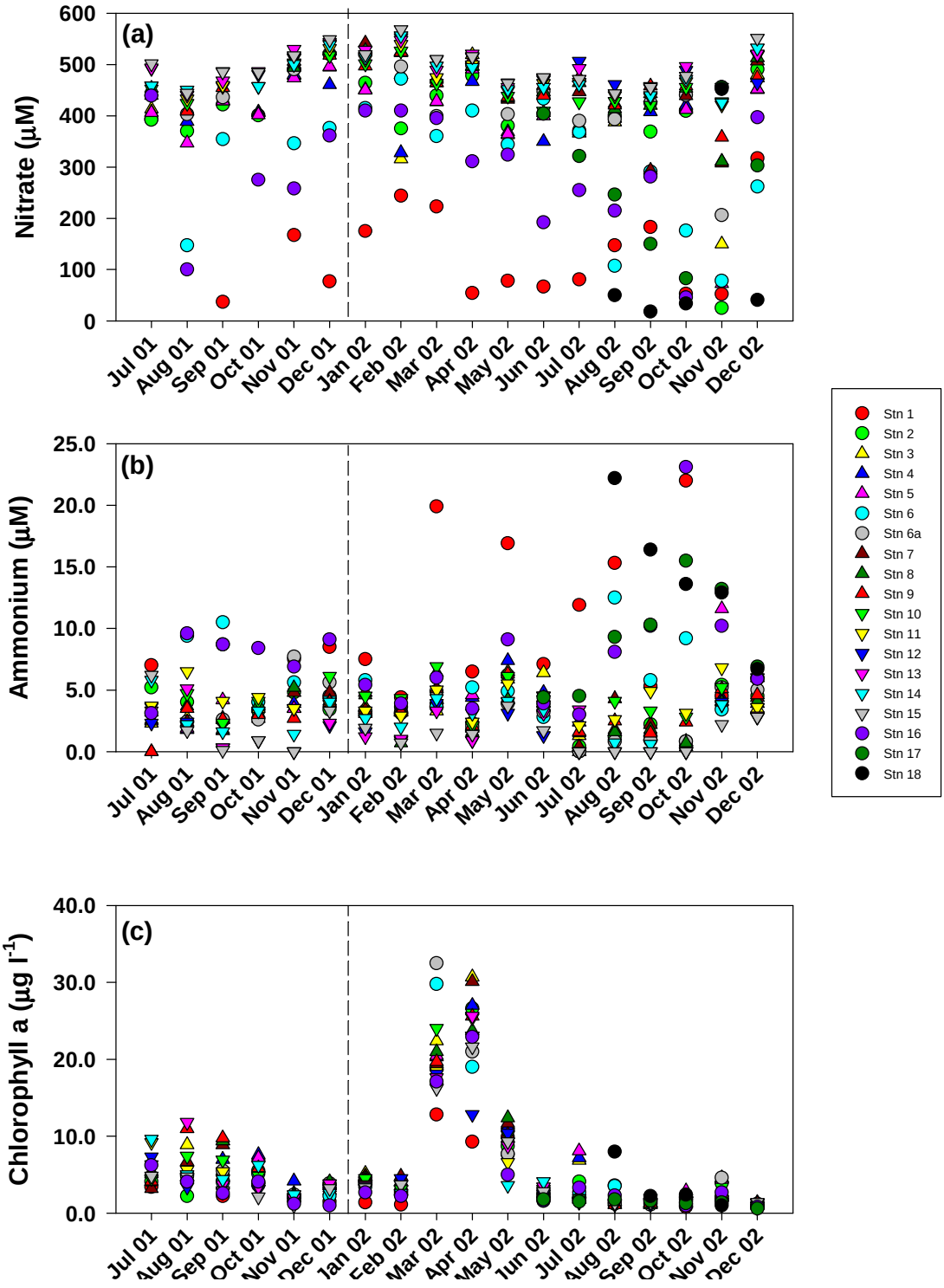

Figure 3.2. Temporal changes in the concentration of (a) nitrate, (b) ammonium and (c) chlorophyll a at all sites. The circular symbol indicates saline samples from sites 1, 2, 6, 6a, 16, 17 and 18, whereas the triangular symbols represents freshwater samples. Ammonium measurements < limit of detection (0.4 µM) were indicated with a concentration of zero.

Ammonium concentrations then increased to a peak in December 2001, and then declined to a second low in February 2002. Throughout 2002 the ammonium concentrations continued to fluctuate with a peak in May and a low in October. A similar variation was seen to that observed in 2001, with increasing ammonium concentrations during November and December 2002. Although the seasonal pattern for ammonium was less clear than for nitrate, high concentrations were observed on a six monthly basis, at the beginning of winter and end of spring, in between which the concentrations declined.

A pronounced seasonal change, with less differentiation between saline and freshwater samples, was observed in the chlorophyll a concentrations (Figure 3.2c). Chlorophyll a concentrations greater than 10 $\mu g\ l^{-1}$ were measured in summer 2001. Following a winter decline, a strong chlorophyll a peak was then observed in March and April 2002, with values up to 30 $\mu g\ l^{-1}$. Following May 2002, the concentrations remained below 10 $\mu g\ l^{-1}$ until the end of the survey period.

These summaries of the entire dataset not only give a first approximation of the seasonal variations in nutrient concentrations, but also indicate that saline and freshwater samples have different concentration ranges. Therefore separation of the freshwater and saline samples is necessary to get a clearer understanding of the seasonal processes within the river and the estuary.

Freshwater nutrient observations A summary of freshwater samples was produced using nutrient data from sites where the river was constrained within only one main channel (sites 9, 10 and 11) or, where the channel was anastomosed, from the site with the highest river discharge (sites 4, 13 and 15). These sites are identified in Figure 3.3 and described in more detail in Table 2.1.

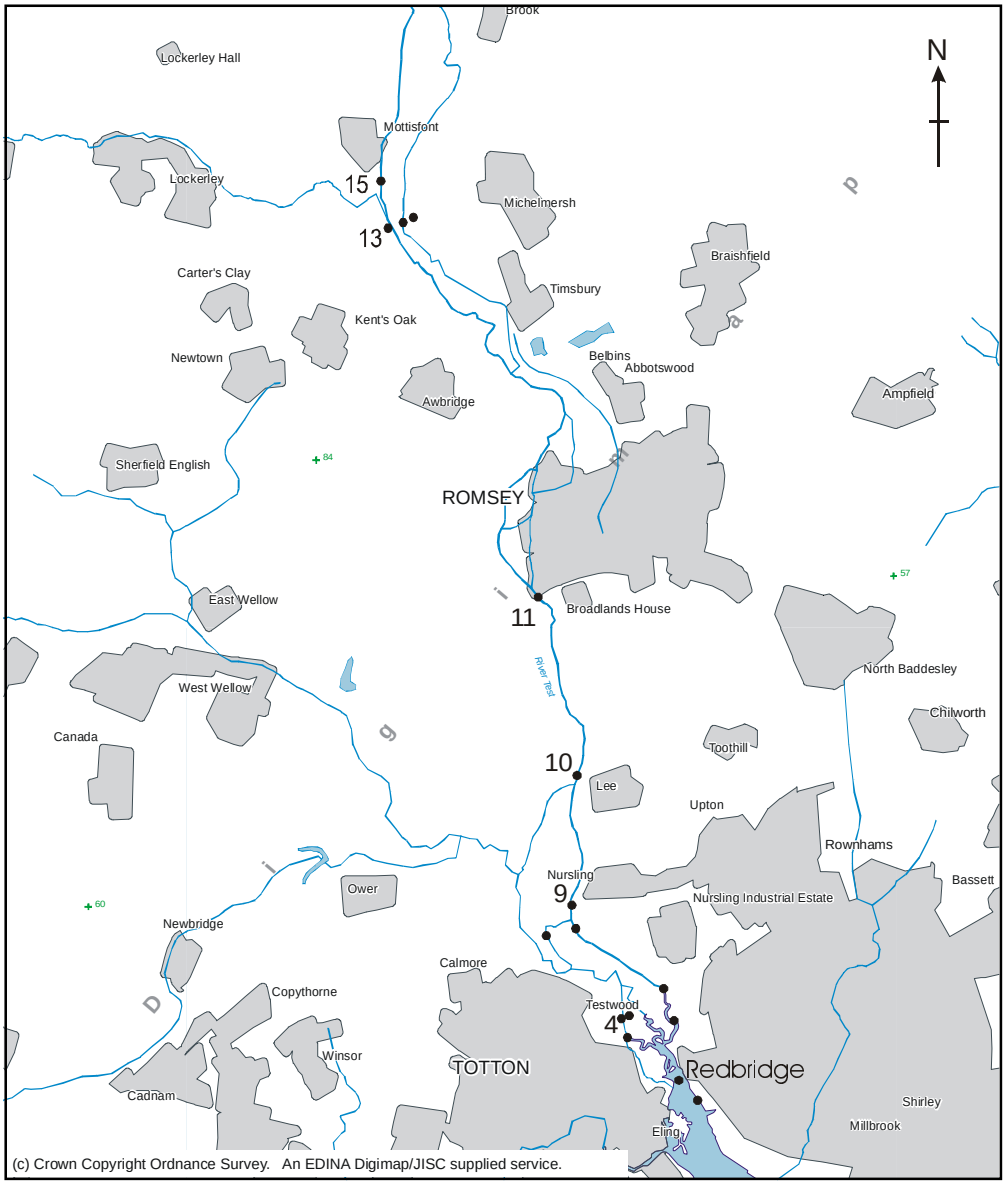

Figure 3.3. Map of main freshwater sites on River Test including both single channel sites (9, 10 and 11) and where several parallel channels were sampled, the channel with the highest flow (4, 13 and 15). Grey shading indicates urban areas.

At these main channel sites in the River Test, nitrate concentrations between 328 µM and 568 µM were observed (Figure 3.4a). This was a much narrower range of concentrations than observed from the full dataset (Figure 3.2a). However, a similar seasonal pattern was apparent. The lowest nitrate concentrations were measured in August 2001, after which the levels increased to a peak in February 2002. The concentrations then proceeded to fall until the lowest median nitrate in August 2001. Towards the end of 2002 nitrate concentrations followed a similar pattern to the previous year.

The ammonium concentrations ranged from the limit of detection for the indophenol blue method (0.4 µM) to a maximum of 7.4 µM in May 2002 (Figure 3.4b). Within any month, there was a wide range of concentrations between sites. Seasonal changes in ammonium concentrations were not as apparent as in the full dataset (Figure 3.2b) and August to December of 2001 and 2002 showed contrasting temporal variations.

DON concentrations at the main channel sites showed no seasonal trends with maximum concentrations of 98 µM measured in December 2002 (Figure 3.4c). From August to November 2001 a gradual increase in median DON concentrations was observed, whereas during the same months in 2002 the DON concentration decreased. During the rest of the study period concentrations fluctuated with some months showing little inter-site variability (May 2002) whereas other months were highly variable (June 2002).

A wide range of dissolved organic carbon (DOC) concentrations were measured in the freshwater samples (8 µM - 575 µM), with a mean concentration of 124 µM. A clear seasonal variation was apparent with spring minima in April and maximum concentrations in the autumn (Figure 3.5a). The timing of the autumn maximum was variable, with 2001 having a peak in August and autumn 2002 having the highest median DOC in November.

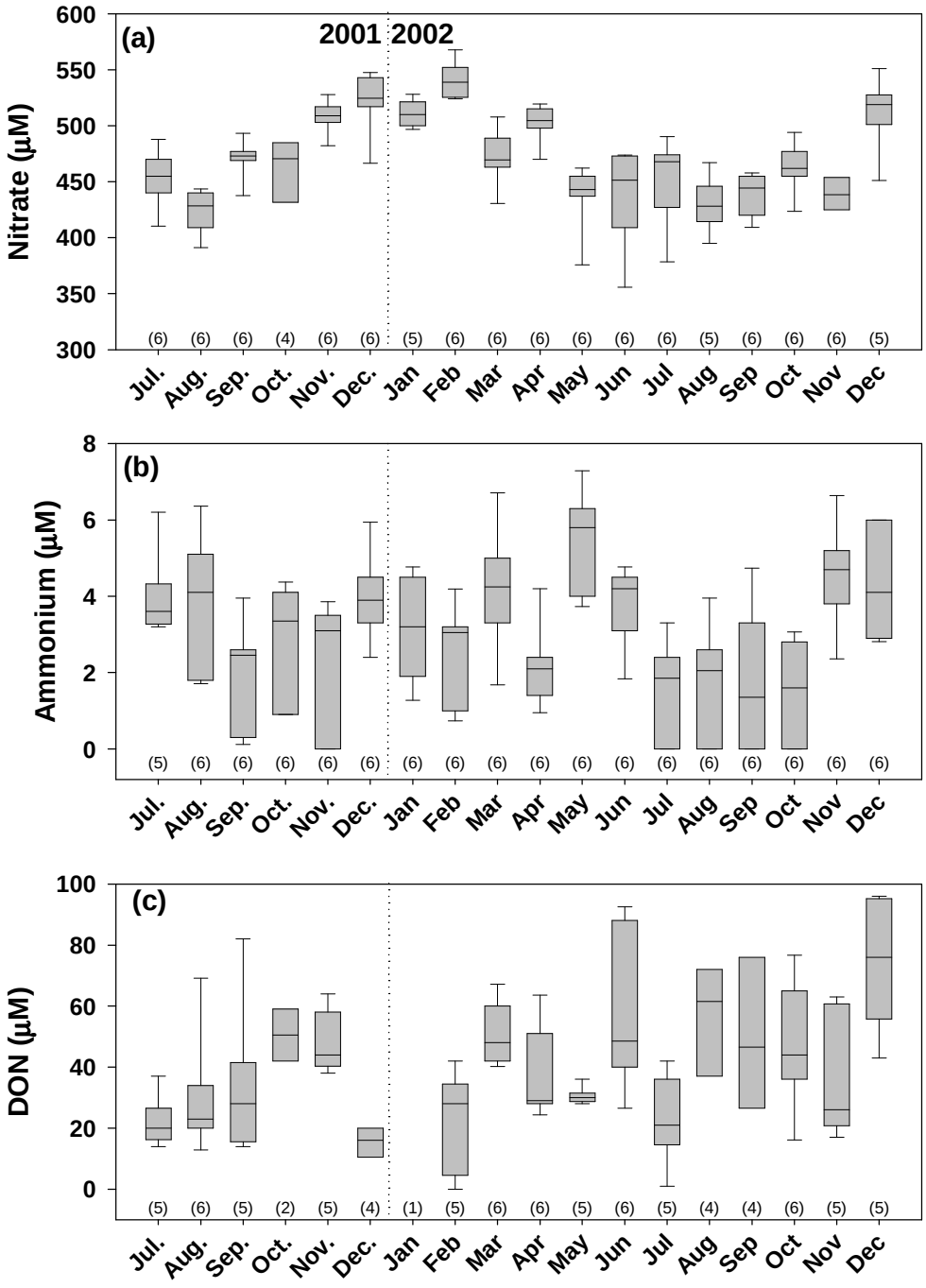

Figure 3.4. Temporal variations in (a) nitrate, (b) ammonium and (c) DON measured in freshwater samples from sites 4, 9, 10, 11, 13 and 15. The upper box boundary represents the 75th percentile and lower, the 25th. The whiskers above and below the box indicate the 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. On the x axis, (number) represents the number of data values for each plot. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability). Ammonium measurements < limit of detection (0.4 µM) are indicated with a concentration of zero.

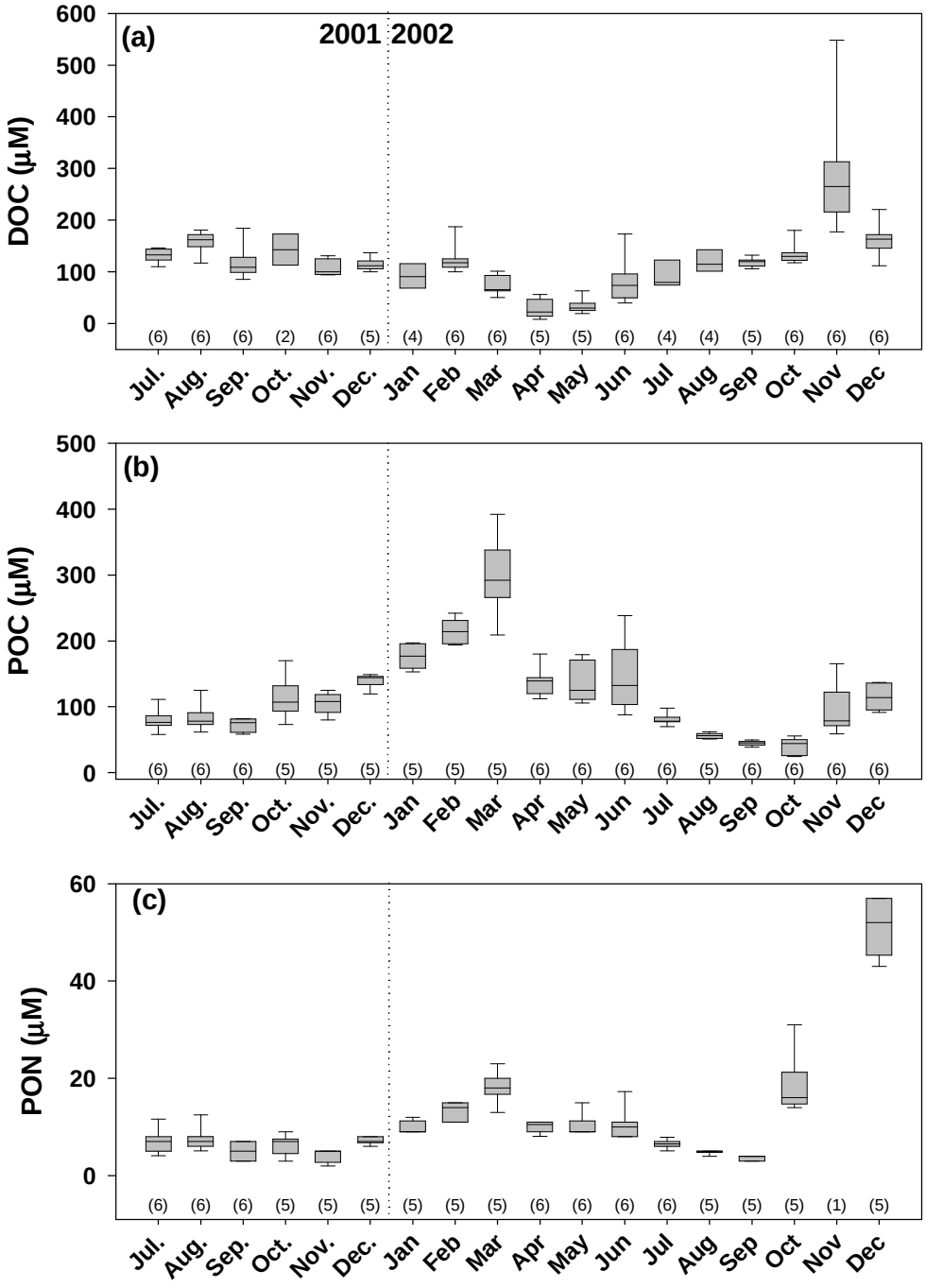

Figure 3.5. Temporal variations in (a) DOC, (b) POC and (c) PON measured in freshwater samples from sites 4, 9, 10, 11, 13 and 15. The upper box boundary represents the 75th percentile and lower, the 25th. The whiskers above and below the box indicate the 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. On the x axis, (number) represents the number of data values for each plot. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

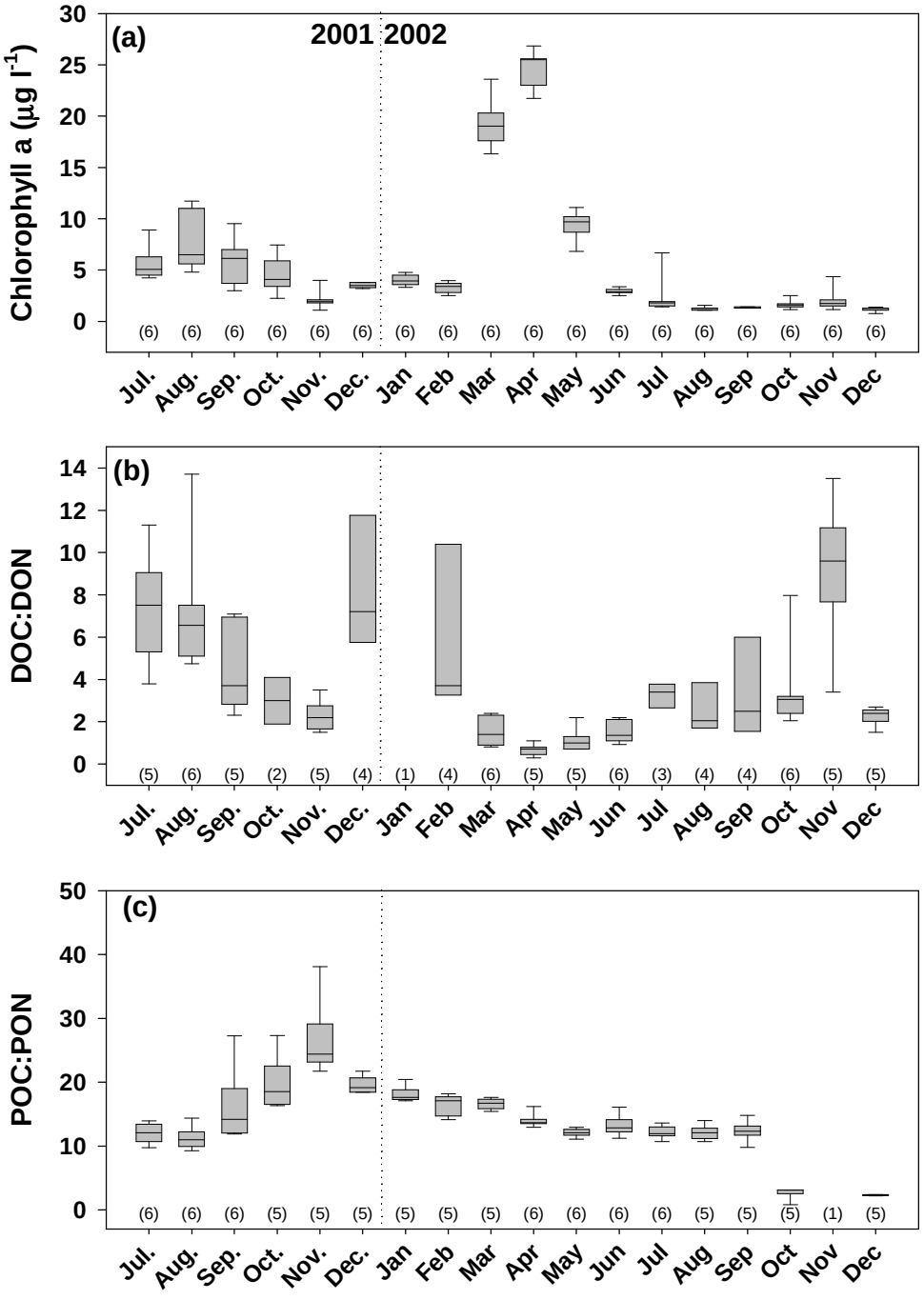

Figure 3.6. Temporal variations in (a) chlorophyll a, (b) DOC: DON and (c) POC:PON measured in freshwater samples from sites 4, 9, 10, 11, 13 and 15. The upper box boundary represents the 75th percentile and lower, the 25th. The whiskers above and below the box indicate the 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. On the x axis, (number) represents the number of data values for each plot. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

The range of particulate organic carbon (POC) concentrations in the River Test was similar to that of DOC (24 – 392 µM), although there was an inverse seasonal variation for POC (Figure 3.5b). Concentrations between July and October 2001 were fairly stable and low, but increased through the winter to a spring peak in March 2002. Following the peak, concentrations dropped rapidly until the autumn when the cycle began again as DOC concentrations increased.

The range of particulate organic nitrogen (PON) concentrations in the River Test was between 3 and 57 µM. The temporal variation closely followed that for POC (Figure 3.5c) with a spring peak of 23 µM in March 2002. However a clear difference was the increase in PON concentrations measured in October and December 2002 which were much higher than the spring increase.

Chlorophyll a concentrations (Figure 3.6a) ranged from 0.7 to 27 µg L^{-1}, but concentrations exceeding 12 µg L^{-1} were only measured in March and April 2002. This marked increase in chlorophyll a in the spring coincided with peaks in POC and PON, whereas other months showed a narrow range of chlorophyll a concentrations. However, July to October 2001 had a greater degree of inter-site variability. There was good overall agreement in the range of data at the 6 freshwater sites (Figure 3.6a) and the whole dataset including measurements of chlorophyll a at all sites (Figure 3.2c).

The ratio of DOC: DON in the River Test had a range of 0.3 to 14.4 (Figure 3.6b). Generally a decrease was observed from July 2001 to April 2002, followed by a gradual increase in DOC: DON to a peak in autumn 2002. April 2002 had the lowest ratio indicating high DON concentrations relative to DOC. Higher ratios were measured in December 2001 and February 2002, which did not concur with the general decline from autumn to spring. A similar anomaly was the high DOC: DON recorded in November 2002.

The POC: PON ratio had a range of 9 to 38 (Figure 3.6c). Throughout the study period a consistent increase in the ratio was observed until a peak in November 2001; this was followed by a gradual decline during spring and summer 2002. The peak in POC: PON in November 2001 was not repeated in 2002, but the ratio was much lower due to the elevated concentrations of PON relative to POC measured in these months.

Seasonal variations in water quality parameters at individual sites This section will focus on data from three sites, located at the upper (site 15) and lower (site 4) limits, and middle (site 11) of the study area to establish whether the location of the freshwater sampling sites influence seasonal changes in nitrate, ammonium, DON and DOC (Figure 2.1). A more detailed consideration of spatial changes in nutrient concentration will be considered in Chapter 4. The daily mean flow at Broadlands, in the middle of the study area has been plotted together with the nutrient concentrations (Figure 3.7) to identify if patterns in the river flow influence the riverine nutrient concentrations (Appendix G).

Seasonal variations in nitrate were clearest at sites 11 and 15 in the mid and upper parts of the study area. Peak concentrations were observed in February and December 2002 during periods of high river flow (Figure 3.7a). At site 4 where lower nitrate concentrations were measured, the relationship between nitrate and flow was less apparent.

There were no obvious seasonal variations in ammonium concentrations in the River Test (Figure 3.7b), as disparities existed between July to December of both 2001 and 2002. There were some similarities between the sites, with elevated concentrations being measured in November and December 2002 during the highest river flow. Fairly low concentrations were measured from August to October 2002 during the low flow period of the year. However, this relationship with flow was not consistent as high ammonium concentrations were detected during the high flows of February 2002. A similar range of concentrations was measured at sites 4 (lower) and 11 (mid). However, further upstream at site 15 generally much lower concentrations of ammonium were detected.

DON concentrations at these three sites showed no clear temporal variation (Figure 3.7c), although there were some similarities in the timing of peaks and troughs between the years. For example increasing concentrations of DON were measured between July and November of both 2001 and 2002. In 2001 this peak was followed by a decrease in concentrations in the winter months, particularly at the mid and upper sampling sites. Gradual increases in concentrations were measured in the spring, peaking in June 2002. There was no overall apparent relationship between river flow and DON concentration.

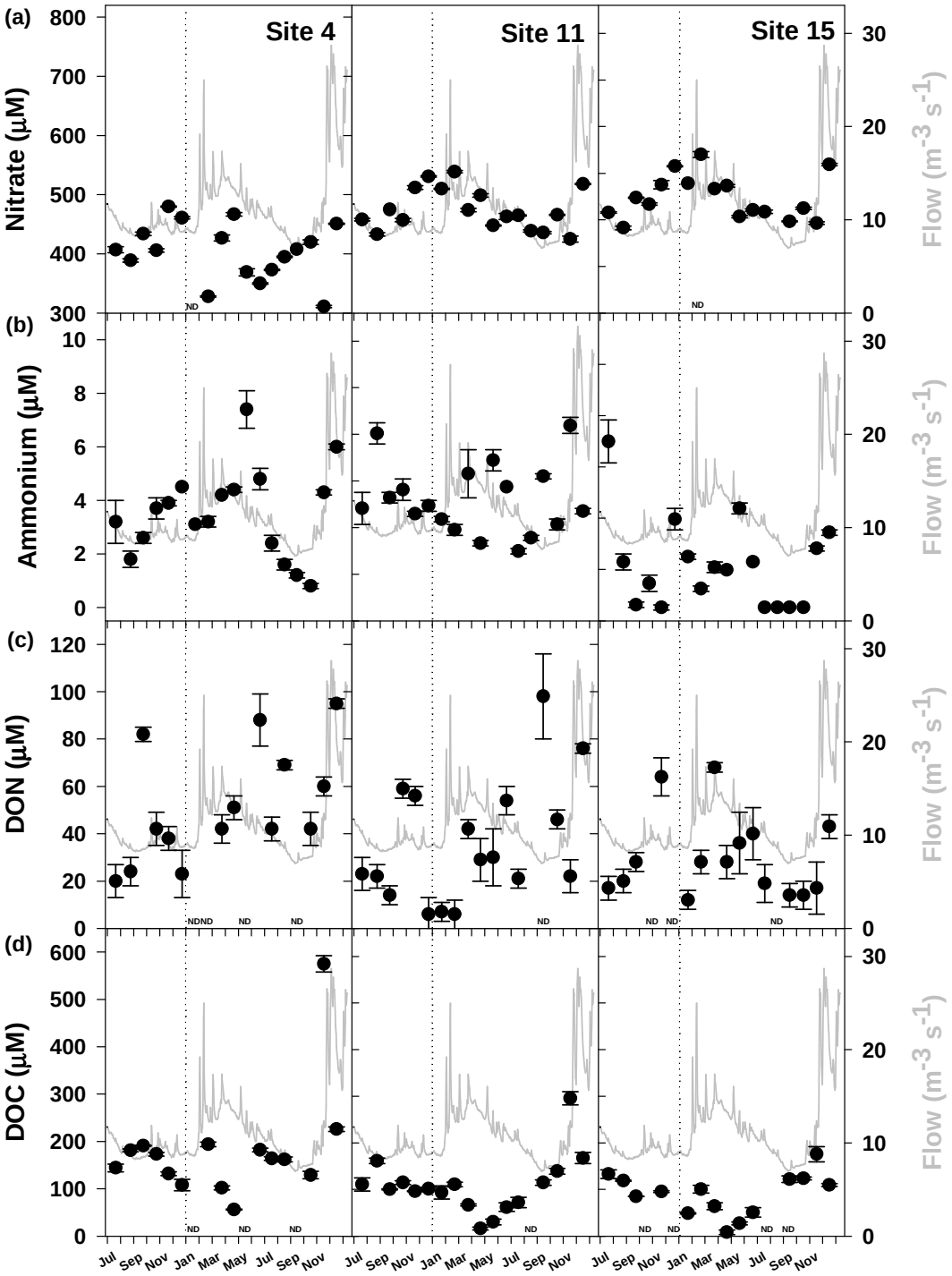

Figure 3.7. Nitrate, ammonium, DON and DOC concentrations from July 2001 – December 2002 at sites 4 (lower Test), 11 (mid-Test) and 15 (upper Test). ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability). Error bars represent one standard deviation. Ammonium measurements < limit of detection (0.4 µM) are indicated with a concentration of zero.

Changes in DOC concentration measured between July and December of both 2001 and 2002 showed little similarity (Figure 3.7d). However, there was strong agreement in the timing of troughs and peaks of DOC concentration throughout the survey area. During the first six months of sampling (July 2001 – January 2002) concentrations of DOC were steadily decreasing in the river. A sharp increase in concentration was observed in February 2002 at all sites, this being most prominent at site 4 in the lower River Test. Concentrations declined sharply in spring 2002 to reach the lowest concentrations in April. DOC increased from these low levels in April to a maximum in November 2002 during the peak flow, which was followed by a sharp decline in December. The patterns of DOC concentration are most similar between sites 11 and 15, and showed little correlation with river flow.

Saline water nutrient observations Water samples collected from freshwater and brackish environments were subject to different environmental conditions, which influenced the nutrient concentrations. Data presented in Figure 3.2 have shown that saline samples contain a different range of nitrate and ammonium concentrations to freshwater samples. Seasonal nutrient variations in the saline samples are expected to reflect the different processes occurring in the estuary. Therefore a summary of the nutrient data is presented (Figure 3.8 to Figure 3.10) to show monthly concentrations of nitrate, ammonium, DON, DOC, POC, PON, chlorophyll a, POC: PON and DOC: DON from sites 1, 2, 6, 6a, 16, 17 and 18.

Nitrate in the saline samples collected from the upper Test estuary and lower River Test had a wide range of concentrations from 21 to 490 µM (Figure 3.8a), which was higher than in the freshwater samples. Prior to June 2002, only 5 of the 7 listed sites were accessible; after this date samples were also collected from sites 17 and 18. The median nitrate concentrations showed low values in August and September 2001 with increasing concentrations in the autumn and winter months. Concentrations gradually declined after a peak was measured in January 2002. The exception to this decreasing trend was July and September, which had the lowest flow conditions. These temporal variations were similar to those seen in the freshwater samples, although the peaks and troughs occurred a month or two later than in the freshwater samples.

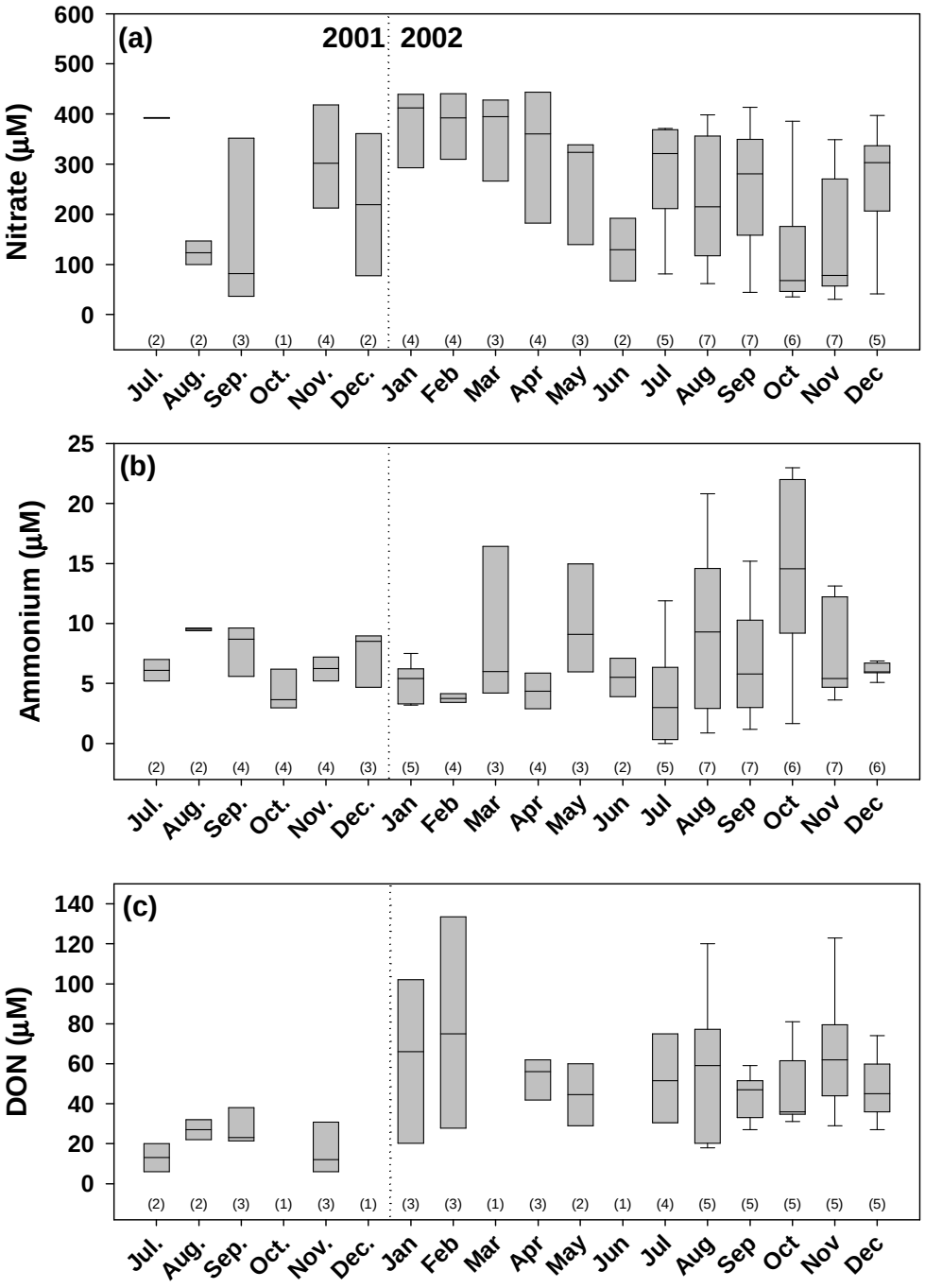

Figure 3.8. Temporal variations in (a) nitrate, (b) ammonium and (c) DON in saltwater samples from sites 1, 2, 6, 6a, 16, 17 and 18. The upper box boundary represents the 75th percentile and lower, the 25th. The whiskers above and below the box indicate the 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. On the x axis, (number) represents the number of data values for each plot. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability). Ammonium measurements < limit of detection (0.4 µM) are indicated with a concentration of zero.

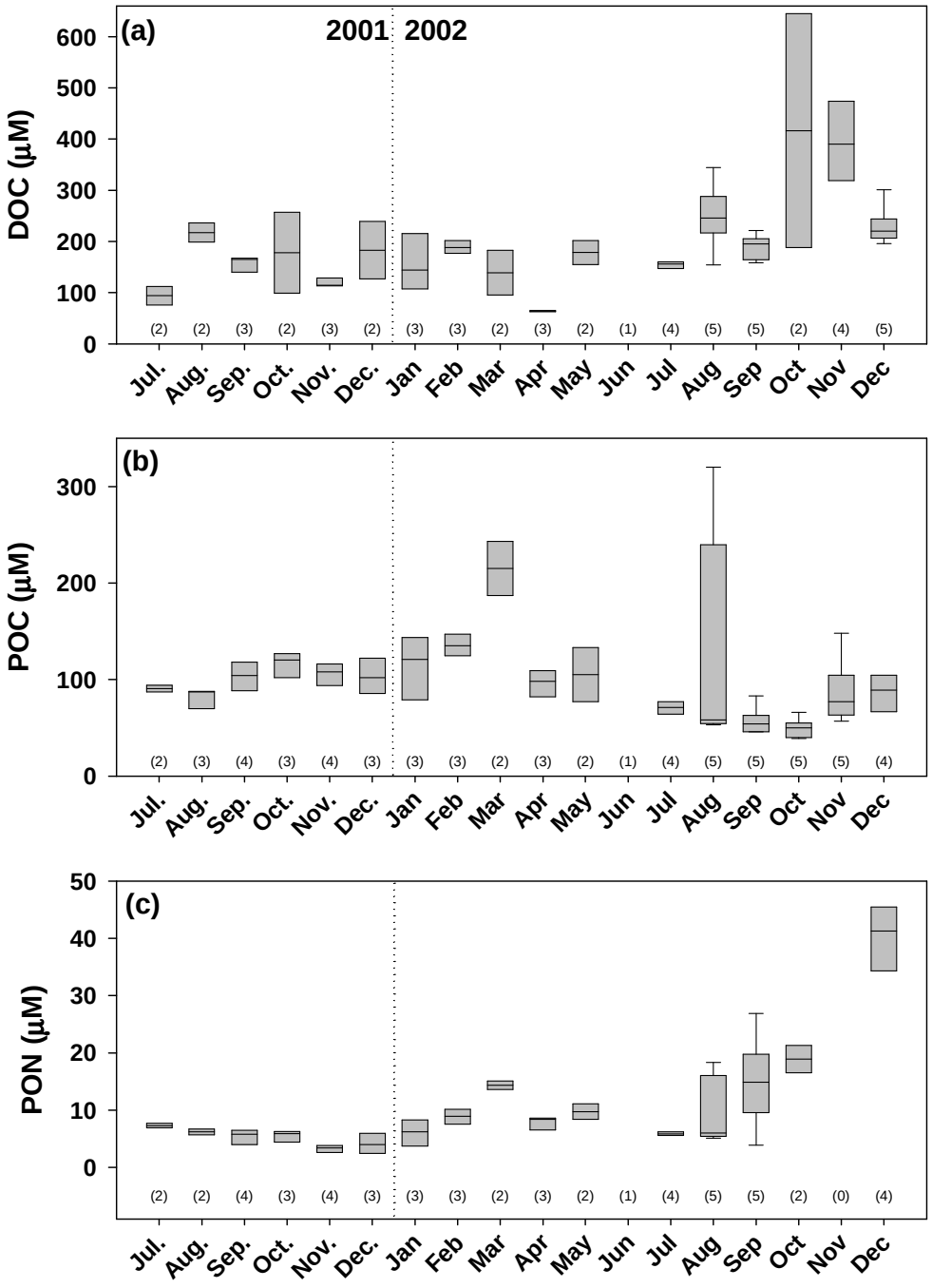

Figure 3.9. Temporal variations in (a) DOC, (b) POC and (c) PON measured in saltwater samples from sites 1, 2, 6, 6a, 16, 17 and 18. The upper box boundary represents the 75th percentile and lower, the 25th. The whiskers above and below the box indicate the 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. On the x axis, (number) represents the number of data values for each plot. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

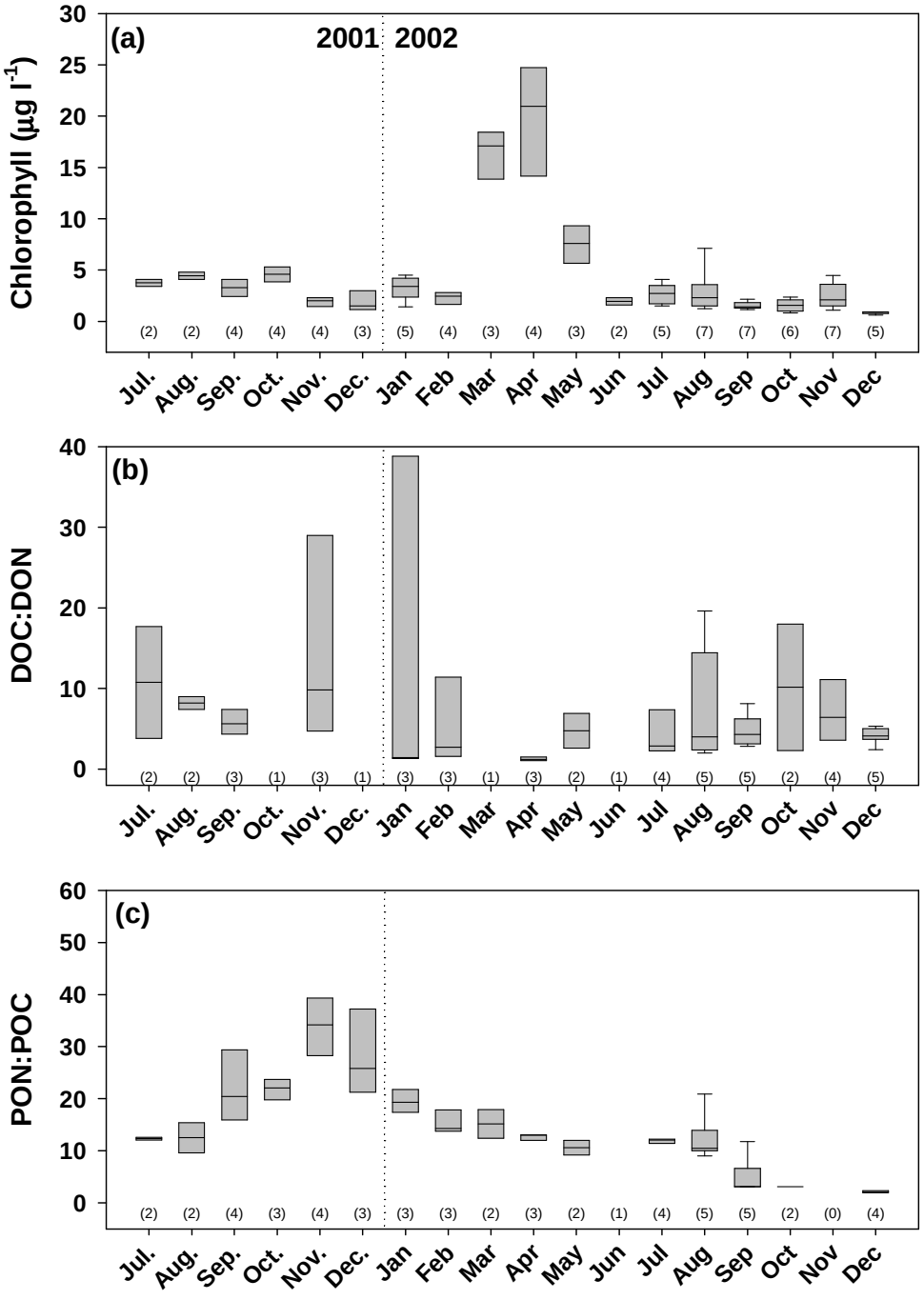

Figure 3.10. Temporal variations in (a) chlorophyll a, (b) DOC: DOC and (c) POC: PON measured in saltwater samples from sites 1, 2, 6, 6a, 16, 17 and 18. The upper box boundary represents the 75th percentile and lower, the 25th. The whiskers above and below the box indicate the 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. On the x axis, (number) represents the number of data values for each plot. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

Ammonium concentrations in the upper estuary are presented in Figure 3.8b. A wider range of concentrations was measured at these saline sites than at freshwater sites, with concentrations ranging from the 0.3 µM to a maximum of 23.1 µM in October 2002. Although no seasonal trend was apparent, similarities were observed between July and December in both years, i.e. an increase in ammonium concentrations was observed until September or October, proceeded by a decline. Fluctuating concentrations of ammonium were measured between January and June 2002 with no consistent trend.

Concentrations of DON in the saline samples (Figure 3.8c) were much higher than in freshwater samples (Figure 3.4c). The highest concentration (199 µM) was measured in June 2002. Although there was no apparent seasonal variation in DON, from July 2001 to February 2002 a steady increase in DON was measured within the saline samples. DON fluctuated throughout 2002, with a particularly wide range of concentrations being measured in January and February 2002. The gradual increase in concentrations measured in 2001 was not observed in 2002.

The measured concentrations of DOC in saline samples were between 62 and 645 µM, although the majority of samples were below 300 µM (Figure 3.9a). Exceptionally high DOC concentrations were measured in October and November 2002. Apart from these months, the rest of the 18 month survey showed some seasonal variation with DOC increasing from July to December 2001 and then falling to a low in April 2002, with a second peak in autumn 2002. There was a high degree of similarity in DOC at all sites and a particularly narrow range of concentrations was measured in saline samples collected in December 2002.

A clear seasonal pattern in POC was observed (Figure 3.9b) which was similar to that seen in the freshwater samples (Figure 3.5). A gradual increase in POC concentrations was measured from September 2001 to March 2002 reaching a peak concentration of 243 µM. This peak was lower than that measured in the freshwater samples (300 µM). The seasonal pattern continued with a decline in concentrations during summer 2002, followed by an increase in November and December 2002. August 2002 was an anomalous month compared to the general decline in concentrations in spring and summer 2002, this was due to high POC concentrations at sites 6 (Testwood Lane) and 18 (Redbridge station deep sample at high tide).

A lower range of concentrations was measured for PON (2 – 48 µM) than POC (Figure 3.9c). The peak PON concentrations in March 2002 were consistent with those measured in the River Test, but the second peak measured in December 2002 was lower. The lowest concentrations of PON were measured in November 2001 and July 2002. Although clear temporal changes in POC concentrations were evident, a cycle of PON was not evident.

Concentrations of chlorophyll a measured in the upper Test estuary were of a similar range to that seen in the freshwater samples (Figure 3.10a). Throughout the year the concentrations were fairly stable, with the exception of spring 2002 when a marked increase in the median concentration was measured. The chlorophyll a concentrations in March 2002 reached 19 µg l^{-1} which was earlier than the peak recorded in freshwater samples in April. Another contrast with freshwater chlorophyll a was the absence of elevated concentrations in August and September 2001.

The range of DOC: DON was 0 to 51 (Figure 3.10b), with no clear temporal variation. Some months (April and December 2002) had consistently low ratios at all sites, whereas other months had a wide degree of variation between sites (November 2001 and January 2002). The high values during these months could be attributed to low DON concentrations relative to high DOC; and the inverse was likely from April to December 2002.

Figure 3.10c shows a clear temporal change in POC: PON ratio, with similar concentrations to those measured in freshwater samples. The lowest ratios (< 10) were observed between September and December 2002 when the PON was especially high. The peak POC: PON ratio occurred in November 2001, which was identical to that in freshwater. The variation in POC: PON was not seasonal, as autumn 2002 did not repeat the increase observed in 2001.

3.3.3 Total nitrogen and total organic carbon composition

The sum of PON, dissolved inorganic nitrogen (DIN) and DON is equal to total nitrogen (TN) and total organic carbon (TOC) is the sum of POC and DOC. Monthly mean concentrations of both TN and TOC and the percentage composition are presented in Figure 3.11 to Figure 3.14. The freshwater and saline samples have been separated using

the same sites as §3.22. The six sites used to characterise the river were 4, 9, 10, 11, 13 and 15 and the seven saline sites were 1, 2, 6, 6a, 16, 17 and 18.

Total nitrogen composition TN concentrations in the River Test during the 18 month survey were between 424 and 641 µM (Figure 3.11a). Concentrations varied little throughout the study period with the exception of December 2002, when markedly higher TN was measured. Elevated concentrations of TN were measured from November 2001 to April 2002 compared to the following six months. It is apparent from Figure 3.11a that the River Test is a nitrate dominated system throughout the year. DON was the second largest form of nitrogen, followed by PON, with ammonium forming the smallest fraction of the TN composition.

Lower TN concentrations were measured in the saline samples (Figure 3.11b) and the TN concentration was more variable in saline than in the freshwater samples. Some temporal variation was apparent in the TN concentrations, which were dominated by nitrate, with peaks occurring in winter 2001 and 2002. The lowest TN was recorded in August 2001, although this was preceded by higher concentrations of TN in July 2001. DON concentrations were higher in the saline than freshwater samples, although the PON and ammonium concentrations were comparable with freshwater samples, particularly in June 2002 when DON was the dominant fraction of TN. There was no clear temporal change in DON, PON or ammonium concentrations.

Figure 3.12a shows the nitrogen species as a percentage of freshwater TN. Nitrate is the dominant species in the river Test ranging from 80 to 95 % of the total nitrogen. DON (with a range of 2 – 12 %) was the second largest fraction providing a mean composition of 7 % of freshwater TN, with ammonium and PON contributing 0.6 % and 2.4 % respectively. No seasonal variation in the percentage TN composition from freshwater samples was apparent.

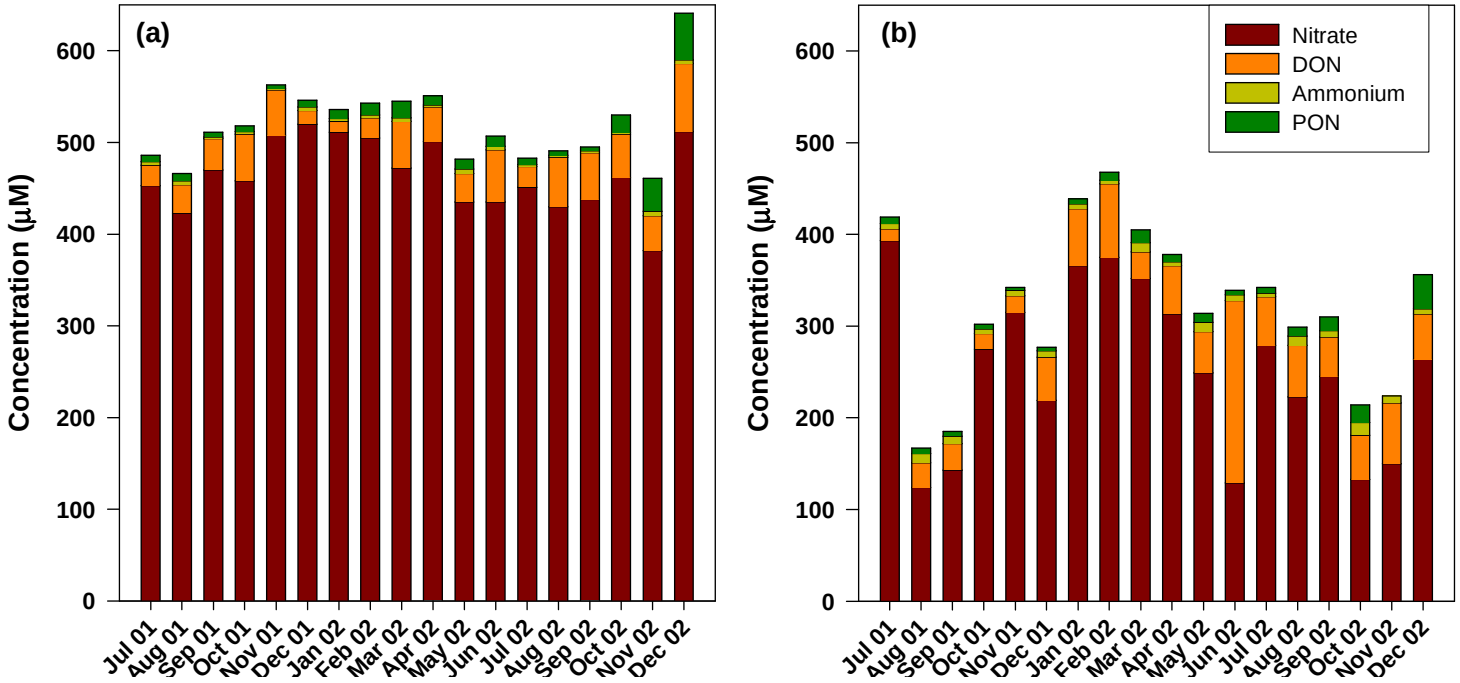

Figure 3.11. Total nitrogen (TN) composition in the (a) River Test (sites 4, 9, 10, 11, 13, 15) and (b) estuary (sites 1, 2, 6, 6a, 16, 17, 18) (July 2001 – December 2002).

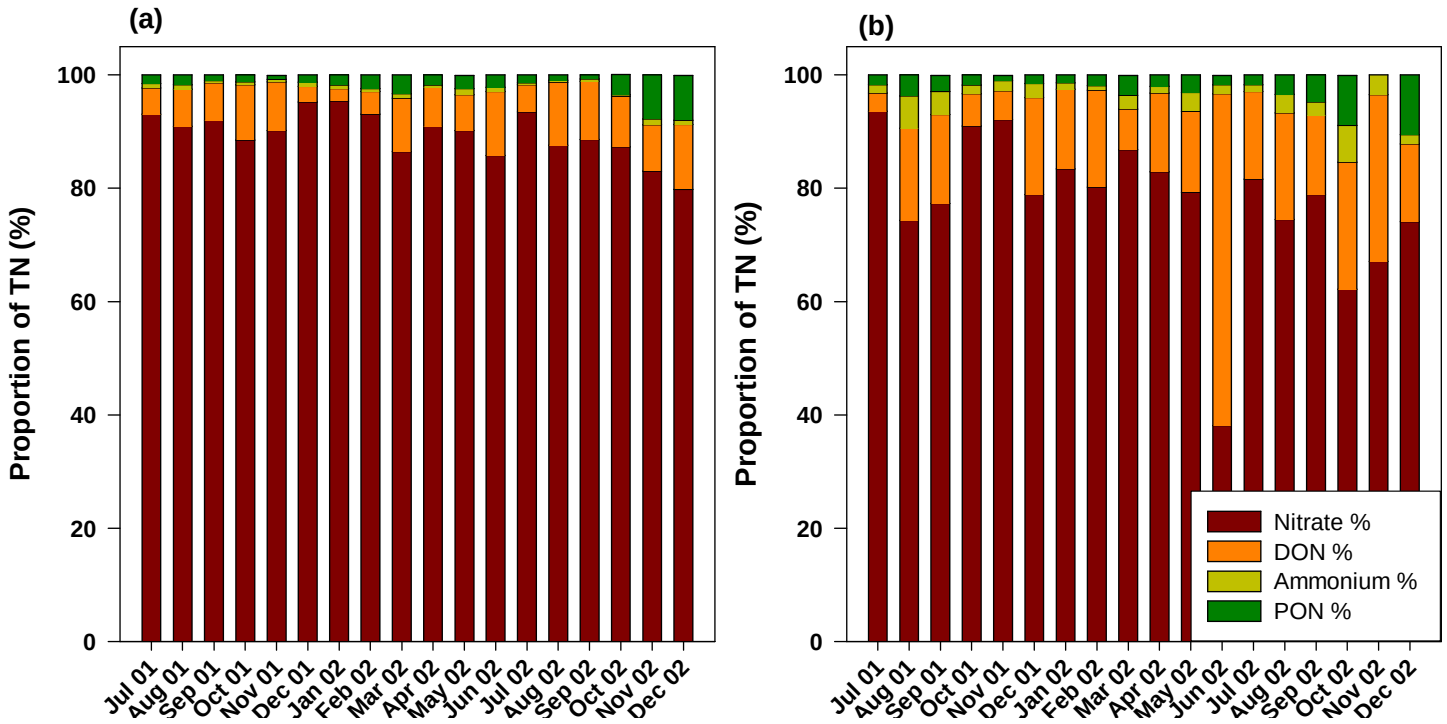

Figure 3.12. Total nitrogen percentage composition in the (a) River Test (sites 4, 9, 10, 11, 13, 15) and (b) estuary (sites 1, 2, 6, 6a, 16, 17, 18) (July 2001 – December 2002).

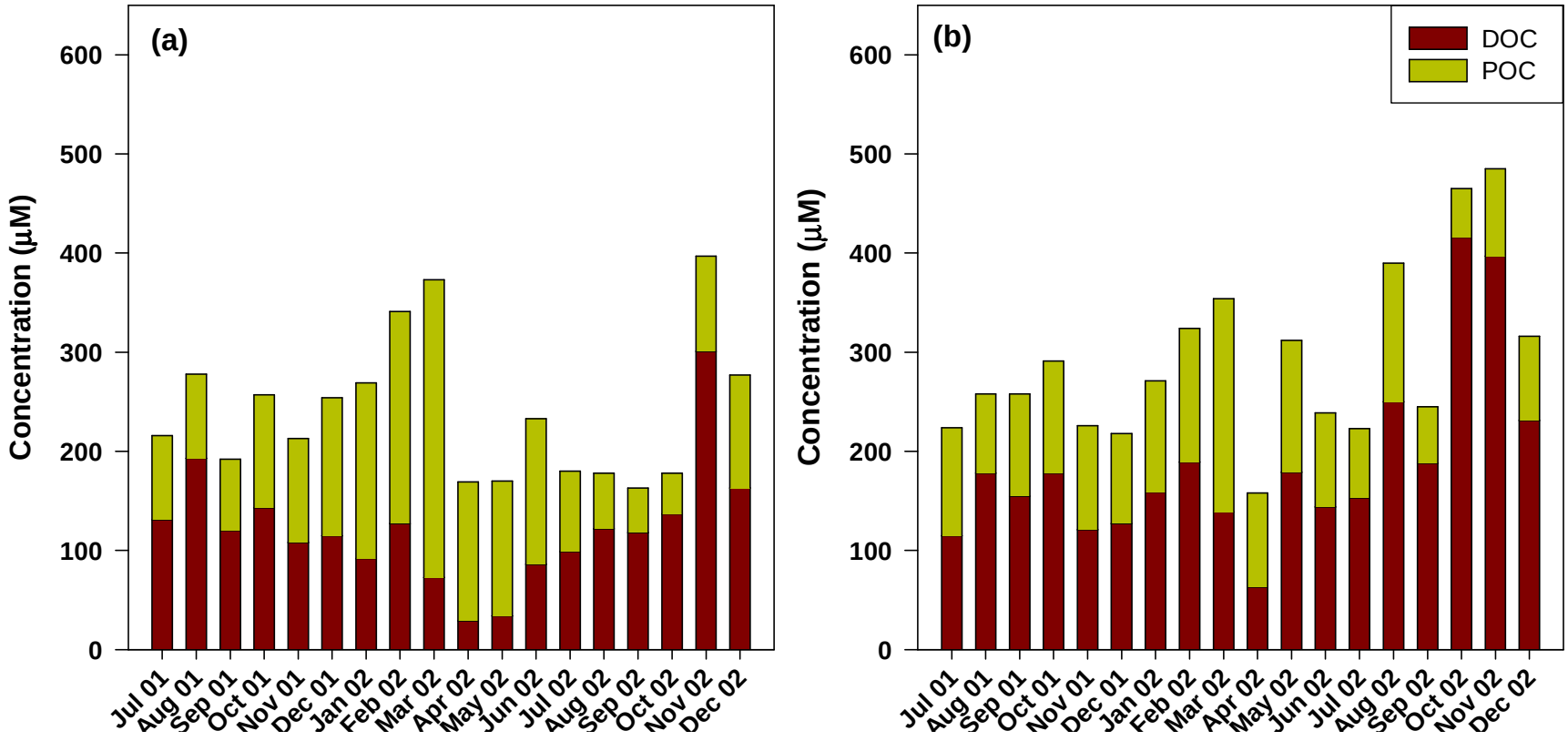

Figure 3.13 Total organic carbon composition in the (a) River Test (sites 4, 9, 10, 11, 13, 15) and (b) estuary (sites 1, 2, 6, 6a, 16, 17, 18) (July 2001 – December 2002

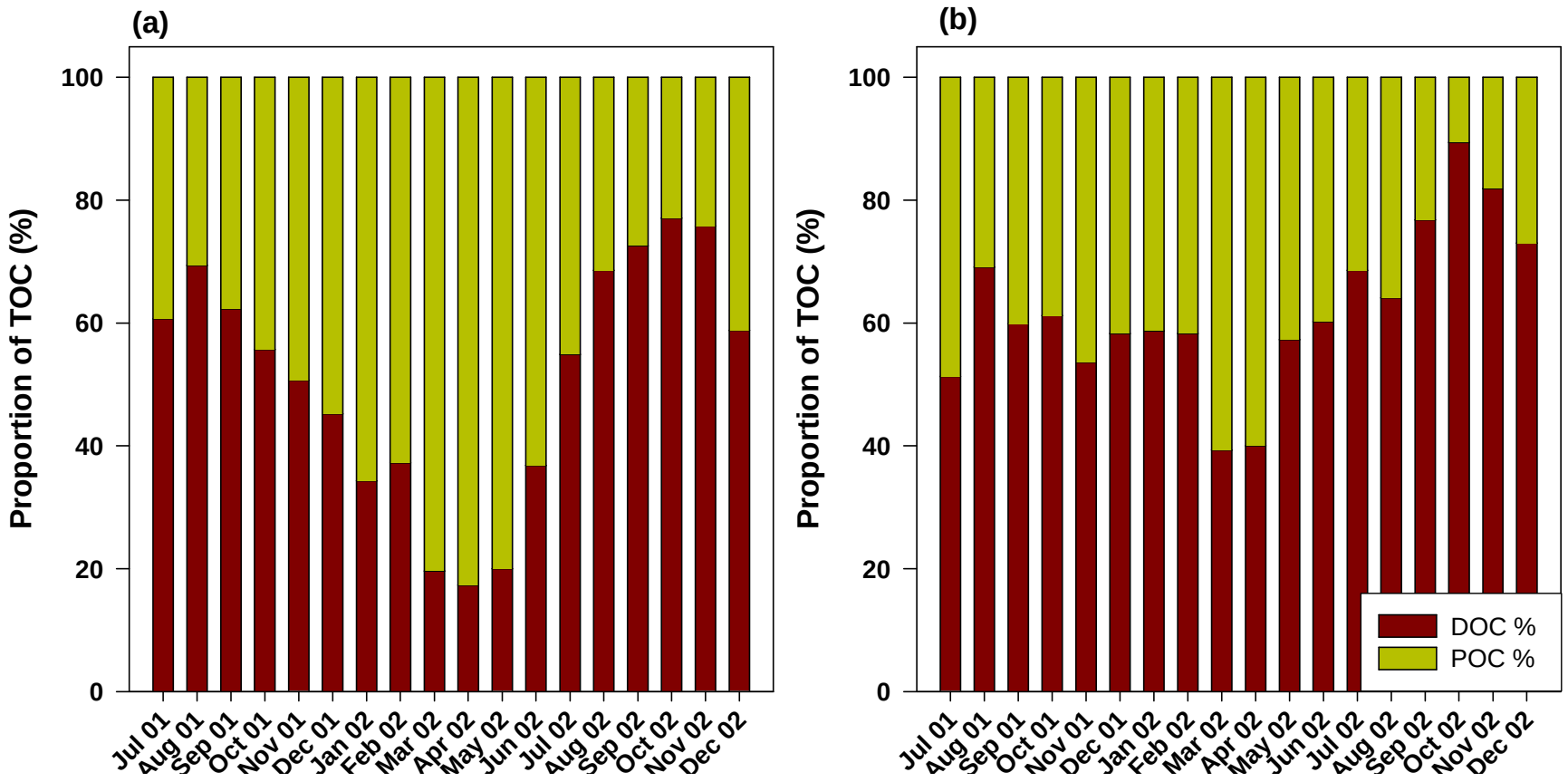

Figure 3.14 Total organic carbon percentage composition in the (a) River Test (sites 4, 9, 10, 11, 13, 15) and (b) estuary (sites 1, 2, 6, 6a, 16, 17, 18) (July 2001 – December 2002)

At the saline sampling sites, DON was a larger proportion of TN than had been seen in the River Test (Figure 3.12b). DON contributed 16.8 % of the TN (although the range was 3 – 59 %), this was almost 50% higher than at freshwater sites. Both ammonium and particulate organic nitrogen also comprised a larger proportion of TN in the saline samples (2.6 % and 3.1 % respectively). Therefore the percentage of TN as nitrate was smaller than in the freshwater samples and had a mean of 77.6 %. There appears to be little temporal variation in the proportion of nitrogen species in either the fresh or saline samples.

Total organic carbon composition DOC and POC concentrations were measured each month in the River Test and upper estuary to determine the composition of TOC. The mean freshwater TOC concentration ranged from 163 to 373 µM (Figure 3.13a). A gradual decline in DOC concentration occurred between autumn 2001 and April 2002, while the POC concentration increased. The inverse was observed towards the later half of the sampling period, when the dominant component of TOC was DOC.

TOC in saline samples had similar concentrations to the freshwater sites, ranging from 158 to 484 µM (Figure 3.13b). Although less apparent, a similar pattern to the freshwater samples could be observed, with POC being the dominant form of TOC in March and April 2002 and DOC for the rest of the year. This decline from peak DOC in the winter to lowest concentrations in spring and summer was less gradual than that seen in the freshwater samples. The largest fraction of TOC for the majority of the sampling period was DOC.

Temporal variations in the composition of TOC were most apparent from the percentage composition shown in Figure 3.14a. A clear decrease in the percentage of DOC in freshwater samples in the spring was followed by an autumnal increase in DOC and vice versa for POC.

The saline samples (Figure 3.14b) were characterised by a more stable composition of TOC with approximately 60 % DOC to 40 % POC. However, some indication of a similar temporal variation was seen as in the freshwater samples. The compositional change in the saline samples was more erratic with less of a gradual transition from the high proportion

of DOC in August 2001 to a low in March and April 2002. DOC was again dominant in October 2002.

3.3.4 Nitrogen loading of the Test estuary

The riverine nutrient inputs to estuaries are determined using measurements of river flow and nutrient concentration. Ideally, daily nutrient measurements would allow characterisation of the full range of flow conditions, but sampling practicalities usually result in the use of monthly nutrient concentrations. Daily river flow data are available for many UK rivers from the Environment Agency. However, the most accurate calculations of nutrient load use flow data from the gauging station close to the tidal limit (Wright, 1980). In the case of the River Test, the anastomosed nature of the lower reaches meant that the closest gauging station that measured the total flow was located at Broadlands (SU 3541 1886), 4 km above the tidal limit (Appendix G). Gauging stations were also located in the lower reaches of the River Test near Testwood on several of the parallel channels and on the River Blackwater tributary (Appendix H). The sum of these flows remains an underestimate of the total flow of the River Test at the tidal limit, as additional water enters the river below the gauging stations from a fish farm and there is accretion of flow below the gauging station at Ower on the River Blackwater, before the confluence with the River Test. Therefore the best approximation of total river flow for the River Test is to correct the flow at Broadlands to accommodate the flow accretion below the gauging station. The total area of the Test catchment based on the interactive digital terrain model at the Centre for Ecology and Hydrology, Wallingford is 1196 km^2, whereas the gauging station at Broadlands only drains 1040 km^2 (Morris and Flavin, 1990).

River Test daily flow Daily river flow data at Broadlands from January 1985 to February 2003 are presented in Appendix I. A prolonged period of river flow in excess of 30 m^{-3} s^{-1} was measured in early 2001, which was associated with flooding in the catchment. For the period of the sampling programme (from July 2001 to December 2002) the mean daily flow was 11 $m^{-3}s^{-1}$. However following the high flows in winter 2001, flow in excess of 15 m^{-3} s^{-1} was only measured between February and April 2002 as well as November 2002 to January 2003.

Figure 3.15a shows the river flow at Testwood and Broadlands for the duration of the monthly sampling survey. The lowest river flows were measured at Broadlands, as the

gauging station was further upstream than Testwood, therefore draining a smaller area of the catchment. The peak flow (in February and November 2002) was therefore higher and more prolonged at Testwood, and marginally later than Broadlands. However the gauging stations at Testwood also received inputs from the River Blackwater, which responds rapidly to precipitation inputs, as the underlying geology is clay and sand in contrast to the permeable chalk bedrock in the majority of the Test catchment (Figure 1.5). Figure 3.15b shows that during the low flow conditions the two areas of the catchment have similar river flow, however at higher flows, the river channels near Testwood maintain a higher water flow for longer than at Broadlands.

Nitrogen load at Broadlands Daily flow data for the duration of the sampling survey (July 2001 – December 2002) in combination with monthly average concentrations of nitrate, ammonium and DON were used to determine the nitrogen load at Broadlands (Walling and Webb, 1985). There are numerous methods for calculating nutrient loads, although many are criticised for averaging the flow data at a very early stage and therefore underestimating the nutrient concentrations at periods of high flow (Walling and Webb, 1981; De Vries and Klavers, 1994; Bukaveckas *et al.*, 1998; Nedwell *et al.*, 2002). The main issue when calculating the load is lack of frequent nutrient measurements in comparison with river flow. Nutrient samples were collected on a monthly basis by the Environment Agency during this study, whereas flow measurements at 15 minute intervals were available from the gauging station at Broadlands. It was therefore necessary to use linear interpolation to estimate the nutrient concentrations between sample collection days. The mean daily nutrient concentration was then estimated using values from 20 days prior and post that calendar day. The daily nutrient load was calculated using the daily mean nutrient concentrations and the mean river flow (Equation 3.1).

The addition of 30 daily nutrient load values gave the total monthly load, and the addition of these monthly load estimates from 12 months of data gave the annual nutrient load for 2002.

$$\text{Load} = \text{daily mean flow} \times [\text{nutrient}] \qquad \text{(Equation 3.1)}$$

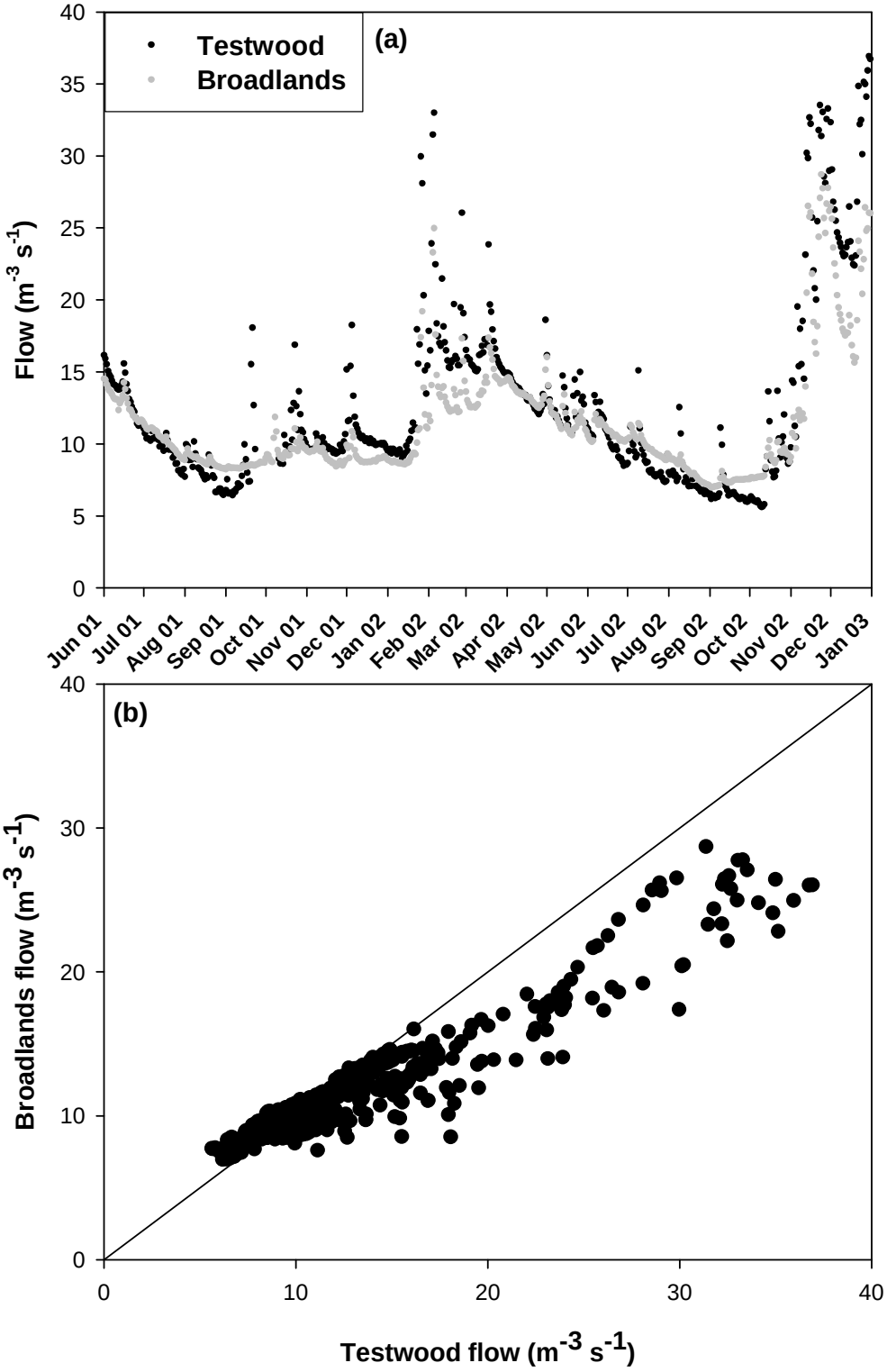

Figure 3.15. (a) Temporal changes in daily mean flow between June 2001 and December 2002 at Testwood and Broadlands gauging stations (b) Daily mean flow at Testwood plotted against Broadlands from June 2001 to December 2002. (Source: Environment Agency). The diagonal line in panel (b) indicates a 1:1 ratio.

Land access constraints prevented the collection of nutrient samples immediately adjacent to the flow gauging station at Broadlands, therefore nutrient samples were collected from site 10 at Longbridge (SU 3547 1786), which was 1.3 km downstream. There were no major inputs of water to the river between these two locations from tributaries or other discharges (Appendix G).

The estimated mean monthly nitrate load at Broadlands was between 9.5 and 30.7 Mmol $mnth^{-1}$ (Figure 3.16a). Temporal changes in nitrate load closely followed the river flow, with the lowest monthly load being recorded in September of both 2001 and 2002 and also a spring peak was measured in February 2002. The highest peak load was recorded in December 2002, which coincided with the peak river flows observed (see Figure 3.15). The annual load of nitrate to the Test estuary in 2002 was estimated to be 206 Mmol y^{-1}.

To make comparisons with other catchments, nutrient loads were calculated per unit area kilometre of the catchment. The area-normalised nitrate load for the River Test / estuary based on nutrient samples collected in 2002 and river flow at Broadlands was 1.7×10^5 mol km^{-2} yr^{-1} (Table 3.1).

Table 3.1. Annual nitrogen load and area-normalised load at Broadlands. NA = data not available.

	Annual load (Mmol yr^{-1})			**Area – normalised load (mol km^{-2} yr^{-1})**		
	Nitrate	**Ammonium**	**DON**	**Nitrate**	**Ammonium**	**DON**
This study	206	2.1	23	1.7×10^5	1.8×10^3	1.9×10^4
EA	212	2.2	NA	1.8×10^5	1.9×10^3	NA

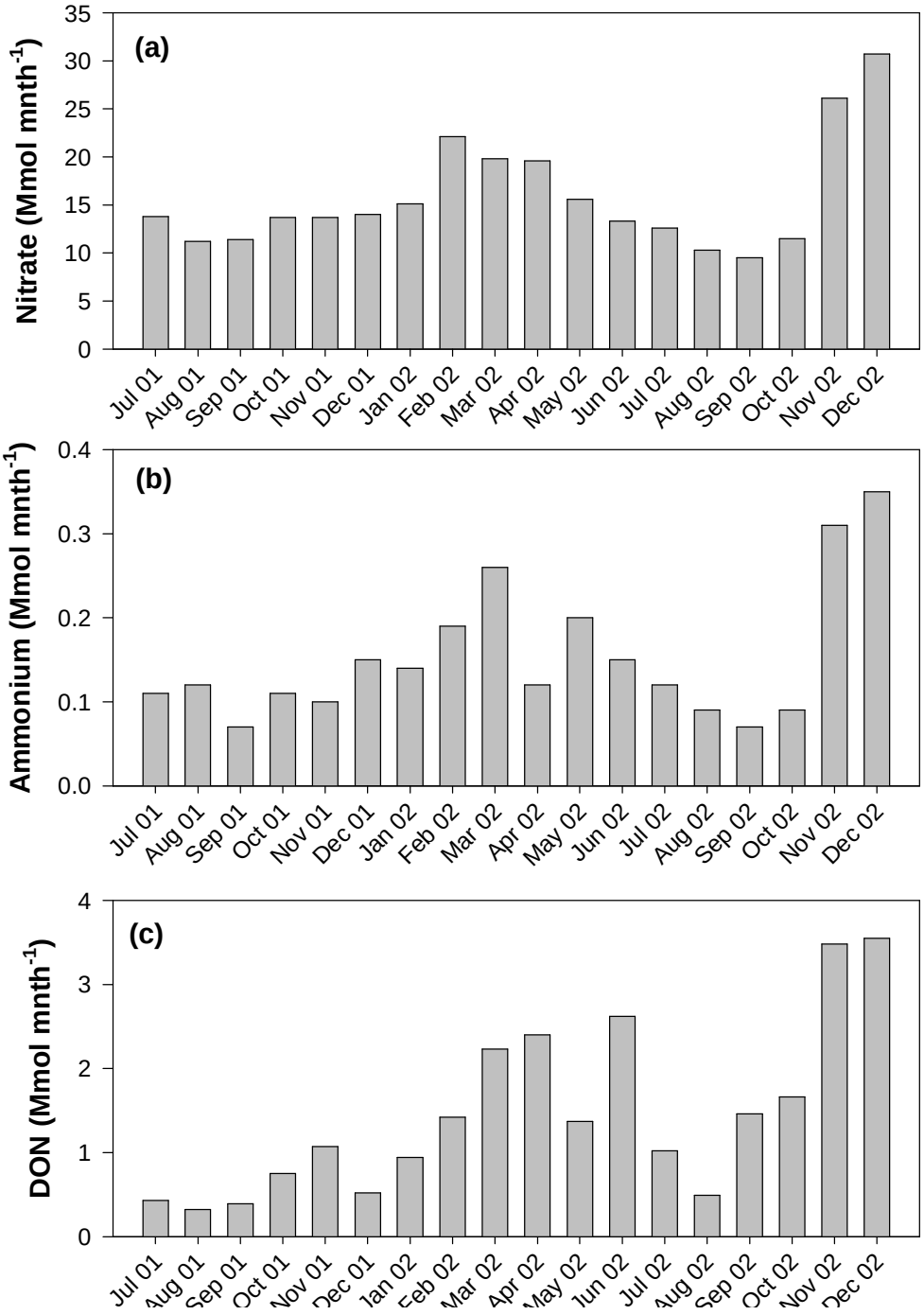

Figure 3.16. Monthly nitrate, ammonium and DON loads calculated using daily flow data from Broadlands gauging station and nutrient concentrations from site 10 at Longbridge.

The ammonium mean monthly load increased more gradually than the nitrate load from September 2001, reaching a peak in March 2002 (Figure 3.16b). With the exception of April 2002, a gradual decline in ammonium load was observed until September 2001, suggesting a seasonal pattern in monthly ammonium load in the River Test. A marked increase was measured in November 2002, reaching a maximum load of 0.35 Mmol $mnth^{-1}$ in December 2002. In 2002, the annual ammonium load was 2.1 Mmol yr^{-1} and the area-normalised load was 1.8 x 10^3 mol km^{-2} yr^{-1}, which was two orders of magnitude lower than the nitrate load (Table 3.1).

Temporal variations between DON and flow were similar to that seen with DIN, but the relationship between mean monthly DON load and river flow was less clear (Figure 3.16c). The spring/early summer peak in DON was recorded in June 2002, which was later than the spring peak observed in both the nitrate and ammonium loads. A similar temporal pattern was apparent to that seen in nitrate and ammonium loads although the DON load was more variable from month to month. The lowest monthly DON load was measured in August of both years (0.32 Mmol $mnth^{-1}$ in 2001 and 0.49 Mmol $mnth^{-1}$ in 2002). This occurred a month before the lowest DIN loads observed in September 2001 and 2002. The highest monthly DON loads occurred in November and December 2002, with a maximum of 3.55 Mmol $mnth^{-1}$ in December. The difference in nutrient load between November and December 2002 was markedly smaller for DON in comparison to DIN. The annual DON load in 2002 was 23 Mmol yr^{-1} and the area-normalised load was 1.9 x 10^4 mol km^{-2} yr^{-1}, which was only one order of magnitude lower than the nitrate load.

Nutrient load from Environment Agency (EA) samples The calculation of nutrient load from EA samples was performed in an identical way to the samples collected during this study. The routine collection of nutrient samples as part of the Harmonised Monitoring Scheme (HMS) included measurements of nitrate, nitrite and ammonium concentrations in the River Test (Appendix J). Samples were collected from the same location as this study at Longbridge (site 10), downstream of Broadlands gauging station. These monthly measurements provide information on the DIN composition; however there have been no measurements of total dissolved nitrogen (TDN), thereby preventing the determination of DON load.

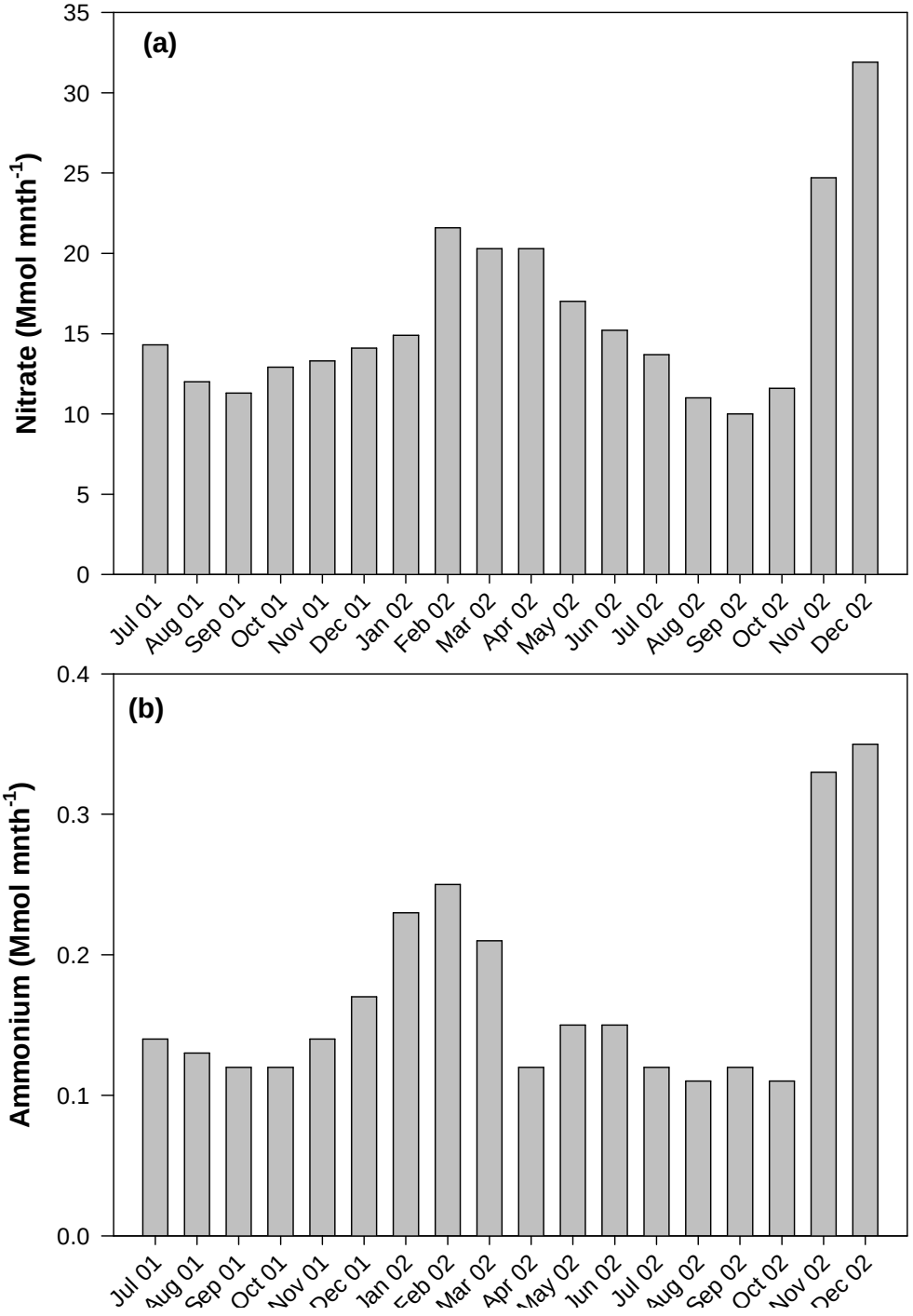

Figure 3.17. Monthly nitrate and ammonium loads calculated using daily flow data from Broadlands gauging station and nutrient concentrations from the Environment Agency collected at Longbridge (SU 3547 1782).

The variation in monthly mean nitrate load from the Environment Agency nutrient samples was virtually identical to that calculated from the nutrients measured in this study (Figure 3.17a). A sustained peak in mean monthly nitrate load was recorded between February and April, followed by a low in September 2002. A large difference was observed between the monthly nitrate load in November and December 2002. The Environment Agency nutrient samples produced an annual nitrate load of 212 Mmol yr^{-1} and area-normalised nitrate load of 1.8×10^5 mol km^{-2} yr^{-1}, both of which were marginally higher than those measured using nutrient data from this study.

The mean monthly ammonium load (Figure 3.17b) was between 0.11 and 0.35 Mmol yr^{-1} and was more responsive to changes in river flow than the nitrate load. For example the peak load increased three fold between October and November 2002, whereas the nitrate monthly load only doubled. The annual ammonium load in 2002 was 2.2 Mmol yr^{-1}, which was higher than that calculated from ammonium concentrations measured in this study. The area-normalised ammonium load was 1.9×10^3 mol km^{-2} yr^{-1}, which was two orders of magnitude lower than that for nitrate.

Nutrient concentration versus river flow The plot of nutrient concentration and river flow presented in Figure 3.18 shows no real relationships for nitrate, ammonium and DON concentrations measured at site 10 at Longbridge. Eighteen months of nutrient samples from this study (nitrate, ammonium and DON) and from January 1997 to December 2002 from Environment Agency sampling programme (nitrate and ammonium) at Longbridge (site 10) and Testwood (site 4) were plotted against the river flow on each sampling date. The increase in nitrate concentration with flow was greatest under low flow conditions, and reached a plateau above 15 m^{-3} s^{-1} (Figure 3.18a). The Environment Agency samples collected over a longer period measured nitrate concentrations under a wider range of flow conditions than observed during this study. The plot of the influence of flow on ammonium concentrations shows no clear relationship. At 8 m^{-3} s^{-1} ammonium concentrations were measured between 3 and 10 µM (Figure 3.18b). Above 25 m^{-3} s^{-1} the concentrations of ammonium stabilised at about 7 µM. Although there are no Environment Agency data available for DON, it is interesting to see there was no obvious relationship between DON concentration and flow (Figure 3.18c). DON showed similar characteristics to ammonium as a wide range of concentrations were measured at any given flow.

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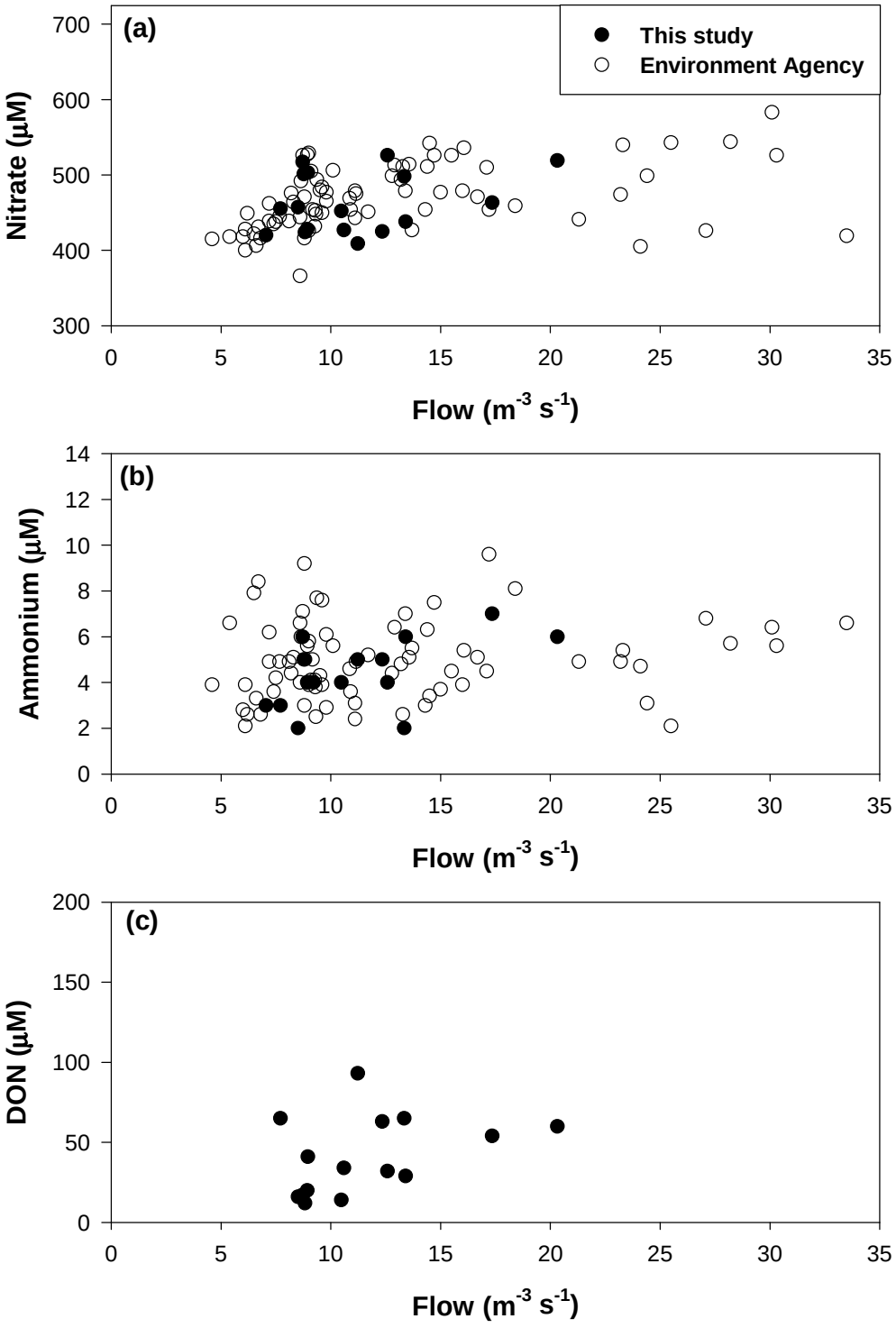


Figure 3.18. River flow at Broadlands and Testwood plotted against nutrient concentrations from site 10 (a) nitrate, (b) ammonium and (c) DON. Environment Agency data was plotted from site 10 (July 2001 to December 2002) and site 4 (January 1997 to December 2002). Note non zero intercept for y axis on panel (a).

3.4 DISCUSSION

3.4.1 Concentrations and proportions of DON and DOC

The River Test is an aquifer fed, nitrate dominated system and, similar to many other lowland British catchments, it is sensitive to changes in nutrient loads. The Test estuary receives a large load of nitrate from the River Test and it is this high flux of nutrients that is believed to contribute to the frequent occurrence of red tides in Southampton Water (Crawford *et al.*, 1997). DON is not routinely measured in the River Test or estuary and few other studies of DON have been carried out in UK river to estuary systems. The second largest dissolved component of TN was DON, measuring a mean of 7 % in the river and 17 % in the Test estuary. This was small relative to the 20 to 50 % received by estuaries, reported in the literature (Seitzinger and Sanders, 1997b) but influenced by the high nitrate concentrations in the river. Despite this, DON remains an important fraction of TN, which is rarely accounted for in nitrogen budgets of estuarine and riverine systems (Seitzinger and Sanders, 1997b).

Mean DON concentrations of 40 µM were measured in the River Test. These are of a similar range to those measured in other UK catchments (Table 3.2), although slightly higher than those recorded in upland regions of Scotland and Wales (Reynolds and Edwards, 1995; Chapman *et al.*, 2001). The areas drained by these catchments are less fertile and have lower population densities than the Test catchment (Nedwell *et al.*, 2002). DON concentrations in the River Test are in good agreement with the review of DON in 32 worldwide studies carried out by Bronk *et al.* (2002) (Table 3.2). Other studies have shown the highest UK riverine DON concentrations were measured in the River Avon (flowing into the River Severn) (Russell *et al.*, 1998).

Marginally higher mean concentrations of DON (47 µM) were measured in the saline samples compared to the freshwater samples. These concentrations were lower than the mean DON concentrations measured previously in Southampton Water, although this study only measured DON in the upper Test estuary and not downstream of the confluence with the Itchen estuary (Hydes and Wright, 1999; Vigus, 2000). The highest percentages were observed in saline samples, although there did not appear to be a positive correlation between DON and salinity (see data presented in Chapter 4).

Study Area	Analysis method	Year of study	DON (µM)		DOC (µM)		Reference
			Estuarine	Riverine	Estuarine	Riverine	
UK							
River Beaulieu, UK	UV	1976 - 1977				142 - 283	(Moore, 1977)
River Test, UK	UV	1976 - 1977				575 - 683	(Moore, 1977)
River Itchen, UK	UV	1974				153 - 180	(Collins, 1978)
River Test, UK	UV	1974				162 - 184	(Collins, 1978)
Welsh upland streams, UK	NK	NK		4 - 5			(Reynolds and Edwards, 1995)
River Dee, UK	PO	1994		14			(Edwards *et al.*, 1996)
River Don, UK	PO	1994		23			(Edwards *et al.*, 1996)
Great Ouse River, UK	UV	1993		21 - 43			(Rendell *et al.*, 1997)
River Avon, UK	HR	1995		106			(Russell *et al.*, 1998)
River Exe, UK	HR	1995		29			(Russell *et al.*, 1998)
River Dart, UK	HR	1995		61			(Russell *et al.*, 1998)
River Severn, UK	HR	1995		55			(Russell *et al.*, 1998)
River Swale, UK	NK	1995 - 1996				250 - 1917	(Eatherall *et al.*, 2000)
River Trent, UK	NK	1993 - 1997			408		(Jarvie *et al.*, 2000)
River Tweed, UK	NK	1993 - 1997			392		(Jarvie *et al.*, 2000)
River Ouse, UK	NK	1993 – 1997			458		(Jarvie *et al.*, 2000)
River Derwent, UK	NK	1993 – 1997			408		(Jarvie *et al.*, 2000)
River Aire, UK	NK	1993 - 1997			458		(Jarvie *et al.*, 2000)
28 Scottish streams. UK	PO	1997 - 1998		13 ± 9			(Chapman *et al.*, 2001)
Southampton Water, UK	UV	1976 - 1977			92 - 458		(Moore, 1977)
Severn Estuary, UK	UV	1976 - 1979			58-183	258 - 658	(Mantoura and Woodward, 1983)
Tamar Estuary, UK	HTCO	1990			100 - 220		(Miller *et al.*, 1993)
Humber estuary, UK	HTCO	1993 - 1995			83 - 1250		(Tipping *et al.*, 1997)
Itchen & Test estuaries & Southampton Water	UV	1995 - 1996	0 – 125				(Hydes and Wright, 1999)
Tamar estuary, UK	HTCO	1990 - 1991			110 - 478		(Miller, 1999)
Itchen & Test estuaries & Southampton Water	HTCO	2000	0 - 186				(Vigus, 2000)

Table 3.2 Summary of DON and DOC concentrations measured in rivers and estuaries. Where UV = ultraviolet oxidation, HTCO = high – temperature catalytic oxidation, C = chemiluminescence, PO = persulphate oxidation, HR = hydrazine reduction and NK = not known. Data are given as maximum and minimum or mean concentrations ± standard deviation. (Adapted and expanded from Bronk *et al.* 2002)

Study Area	Analysis method	Year of study	DON (µM)		DOC (µM)		Reference
			Estuarine	Riverine	Estuarine	Riverine	
Itchen & Test estuaries & Southampton Water	HTCO	2000	0 - 186				(Vigus, 2000)
Yealm estuary, UK	HTCO	2002	2 - 58		100	225	(Bahr *et al.*, 2003)
Tyne estuary, UK	HTCO	2002 - 2003			427 - 1427	275 - 1275	(Baker and Spencer, 2004)
Ouse – Humber estuary, UK	HTCO	1995			10 - 110	394 – 474*	(Alvarez-Salgado and Miller, 1998)
This study (River Test and estuary	HTCO	2001 - 2002	0 - 199	0 - 98	62 - 645	20 - 575	
Europe							
Summary of 15 European Rivers	NK	NK				117 - 967	(Thurman, 1985)
Tagliamento River, Italy	HTCO	1999 - 2000				34 - 83	(Kaiser *et al.*, 2003)
Boreal streams, Sweden (in spring flood)	Urea	1998		16.6 – 36.4			(Stepanauskas *et al.*, 2000)
Three wetlands, Sweden	PO	1996		13 - 180			(Stepanauskas *et al.*, 1999)
Elbe estuary, Germany	PO	1996	49 - 79				(Kerner and Spitzy, 2001)
USA							
Delaware River	HTCO	1992		4.7 – 46.5			(Seitzinger and Sanders, 1997)
Hudson River	HTCO	1992		27.8 – 33.5			(Seitzinger and Sanders, 1997)
Altamaha River	NK	1974 - 1993		25		668	(Alberts and Takacs, 1999)
Black River	NK	1974 – 1993		46		1056	(Alberts and Takacs, 1999)
Edisto River	NK	1974 – 1993		29		7709	(Alberts and Takacs, 1999)
Ogeechee-Eden River	NK	1974 – 1993		37		747	(Alberts and Takacs, 1999)
Ogeechee-Oliver River	NK	1974 – 1993		28		716	(Alberts and Takacs, 1999)
Peedee River	NK	1974 – 1993		33		493	(Alberts and Takacs, 1999)
Satilla River	NK	1974 – 1993		54		1588	(Alberts and Takacs, 1999)
Savannah River	NK	1974 – 1993		24		419	(Alberts and Takacs, 1999)
St Marys River	NK	1974 - 1993		47		2328	(Alberts and Takacs, 1999)
White Mountain streams, New Hampshire	HTCO & C	1996 - 1997		14		500	(Goodale *et al.*, 2000)

Table 3.2. Continued.* means measured during an *Aureococcus anophagefferens* bloom

Study Area	Analysis method	Year of study	DON (µM)		DOC (µM)		Reference
			Estuarine	Riverine	Estuarine	Riverine	
Delaware River	HTCO	1996				218	(Mannino and Harvey, 2000)
Mississippi and Atchafalaya River plumes	HTCO	July 1993	8 - 22	13 - 27			(Pakulski *et al.*, 2000)
Paine Run, Virginia	HTCO	1997		2.3 ± 1.4		105 ± 49	(Buffam *et al.*, 2001)
Apalachicola River	PO	1994 - 1995	13	1 - 14			(Mortazavi *et al.*, 2001)
Saddle stream, Colorado	PO	1996 - 1998		0 - 15		50 - 270	(Williams *et al.*, 2001)
Pine Barrens, NJ.	HTCO	1999				2500	(Maurice and Leff, 2002)
Neversink River, Catskill Mountains	PO	1997				80 ± 7	(Sobczak *et al.*, 2002)
Cascade Mountains	Kjeldahl	1969 - 2001		1 - 3			(Vanderbilt *et al.*, 2002)
Chena River, Alaska	HTCO	2001				148	(Guo *et al.*, 2003)
Rocky Mountain streams	PO & HTCO	1999 - 2001		8 - 9		458	(Kaushal and Lewis Jr., 2003)
Tomales Bay	UV	1987 - 1989	4.8 – 12.6	3.9 – 17.9			(Smith *et al.*, 1991)
Chesapeake Bay	PO	1990	22				(Bronk, D. A. and Glibert, P. M., 1993)
Chesapeake Bay	PO	1990		26			(Bronk, D. A. and Glibert, P.M., 1993)
Ocklockonee estuary	HTCO	1994			189 - 1570		(Powell *et al.*, 1996)
Galveston Bay	HTCO	1993 & 1995			160 - 495		(Guo and Santschi, 1997)
Chesapeake Bay	HTCO	1994			118 - 215		(Guo and Santschi, 1997)
Chesapeake Bay	PO	1989 - 1991			292 ± 13		(Fisher *et al.*, 1998)
Columbia estuary	HTCO	1995			160		(Hopkinson *et al.*, 1998)
Susquehanna estuary	HTCO	1995	23		213		(Hopkinson *et al.*, 1998)
Santilla estuary	HTCO	1995	59		1700		(Hopkinson *et al.*, 1998)
Parker estuary	HTCO	1995	26		594		(Hopkinson *et al.*, 1998)
Waquoit estuary	HTCO	1995	40		630		(Hopkinson *et al.*, 1998)
San Francisco Bay	UV	1996			52 - 172		(Murrel and Hollinbaugh, 2000)
Inter laboratory study, Lewes Harbour, Delaware Bay	PO, UV, HTCO,	2000	23 ±5				(Sharp *et al.*, 2002)

Table 3.2 Continued

Study Area	Analysis method	Year of study	DON (µM)		DOC (µM)		Reference
			Estuarine	Riverine	Estuarine	Riverine	
Winyah Bay	HTCO	1998 - 1999			325 - 1142	708 - 1808	(Goñi *et al.*, 2003)
Pawcatuck River estuary	PO	1993 - 1995			270 - 796	113 - 210	(McKenna, 2004)
Worldwide							
Summary of unpolluted rivers and streams	NK	NK				83 - 700	(Meybeck, 1982)
Summary of unpolluted rivers and streams	NK	NK		4 - 79			(Meybeck, 1982)
Summary of polluted rivers and streams	NK	NK		30 - 106			(Meybeck, 1982)
Summary of 15 rivers	NK	NK				250 - 3333	(Thurman, 1985)
Summary of 20 rivers	NK	NK				83 - 2083	(Spitzy and Leenheer, 1991)
Unpolluted rivers and streams	NK	NK		4 - 71			(Meybeck, 1993)
Rivers affected by human activities	NK	NK				150 - 567	(Meybeck, 1993)
Amazon River, Brazil	HTCO	1990			231 - 647		(Amon and Benner, 1996)
Rio Negro, Brazil	HTO	1993				801	(Amon and Benner, 1996)
Rio Solimoes, Brazil	HTO	1993				378	(Amon and Benner, 1996)
Lena River, Siberia	HTCO	1989 – 1991			830	850	(Cauwet and Sidorov, 1996)
Canadian Rivers	UV	1983 - 1989				250 - 2500	(Clair *et al.*, 1996)
Lena river, Siberia	PO	1994		9 - 28		300 - 1000	(Lara *et al.*, 1998)
Amazon River, Brazil	HTCO	1994				78 - 346	(Hedges *et al.*, 2000)
Furo do Meio mangrove creek, Brazil	PO & HTCO	1996 - 1997	20		360		(Dittmar and Lara, 2001)
Review of 32 riverine studies	PO, HTCO, NK	1987-1999	22.5 ±17.3	34.7 ± 20.7			(Bronk, 2002)
Rio Negro, Brazil	HTCO	1997				704	(Moreira-Turcq *et al.*, 2003)
Rio Solimoes, Brazil	HTCO	1997				278	(Moreira-Turcq *et al.*, 2003)

Table 3.2 Continued

A study of nutrients in Southampton Water showed spatial variation in the percentage of DON, with up to 40 % TDN at Dockhead at the confluence of the two river estuary (Hydes and Wright, 1999). As the present study only included the tidal reaches these spatial variations in the lower estuary were not apparent. Few studies have been carried out to determine DON concentrations in other UK estuaries as well as other estuaries worldwide (Table 3.2)(Bronk, 2002; Bahr *et al.*, 2003). At present the author is aware of only one other published study of DON concentrations in a UK estuary (Bahr *et al.*, 2003). This investigation was carried out on the Yealm and measured similar concentrations to those observed in this study.

A higher proportion of TOC as DOC was measured in saline than in freshwater samples (62 % and 51 % respectively). This was also reflected in higher concentrations of DOC in the saline (62 – 645 µM) than in freshwater samples (8 – 575 µM). Compared to other riverine studies of DOC concentrations, the range was similar to that measured in the River Severn, and lower than the River Tyne which drains carbon-rich peat uplands (Table 3.2) (Mantoura and Woodward, 1983; Baker and Spencer, 2004). Concentrations of DOC in the River Test were towards the lower end of the range measured in worldwide rivers, which may be associated with the geology and land use (Clair *et al.*, 1996; Buffam *et al.*, 2001). The highest concentrations worldwide were from rivers draining forested and agricultural catchments, suggesting that terrigenous inputs were important (Clair *et al.*, 1996; Alberts and Takacs, 1999). A study of the River Swale in Yorkshire, however, found that high soil organic carbon content did not necessarily result in high DOC loads (Eatherall *et al.*, 2000).

The saline samples in the Test contained higher concentrations of DOC than freshwater samples. Higher concentrations of DOC also occur in the Tyne estuary than in the Tyne river (Table 3.2) (Baker and Spencer, 2004). The Tyne estuary receives large anthropogenic inputs from sewage treatment works, which are likely to increase the DOC concentrations (Baker and Spencer, 2004). The Test estuary had similar concentrations of DOC compared to other studies of estuarine DOC.

3.4.2 Temporal variations in dissolved organic matter

The concentrations of DON and the proportion of DON as a percentage of total nitrogen showed no consistent seasonal trends. In addition there was a lack of consistent temporal variation between DOC and DON. Similar observations have been reported in a high elevation catchment in Colorado, USA in a study of riverine DON (Williams *et al.*, 2001).

Nitrate was the dominant nitrogen species in both the River Test and estuary. A strong seasonal variation was apparent with peak concentrations being measured during the winter months, coinciding with high rainfall and resulting in rapid runoff and so a transfer of terrestrially derived nitrate to surface waters (Meybeck *et al.*, 1990; Goodale *et al.*, 2000). The lowest concentration occurred in the spring to summer months and was associated with phytoplankton production during the growing season (Hydes and Wright, 1999; Goodale *et al.*, 2000). Even though DON was the second largest dissolved nitrogen component, no similarities in temporal variations were seen when compared with nitrate. Previous studies of Southampton Water have observed large seasonal changes in nutrient concentrations (Kifle and Purdie, 1993; Iriarte and Purdie, 1994). Concentrations of DON, DOC and ammonium were higher in saline than freshwater samples but no obvious seasonality was observed.

The first research to suggest seasonal variations in DON was carried out using data collected over an 11 year period in the English Channel (Butler *et al.*, 1979). An increase in concentrations was observed from January to August, followed by a steady decline until December. The long-term aspect of this dataset provides evidence of seasonal patterns in DON concentrations. Although this study was not carried out in a river or estuarine environment, other studies have shown either a presence or lack of seasonal variation in DON concentrations (Table 3.3). This distribution of studies highlights the lack of worldwide research into DON seasonal variation with most of the research focusing on systems in temperate latitudes of the northern hemisphere.

The lack of seasonal variation in DON concentrations in this study aligns with other studies (Bronk, 2002). The cycling of DON on a temporal scale is clearly more complex than for dissolved inorganic nitrogen species. Once a better characterisation of the chemical composition of DON has been determined this should be used to identify seasonality in components of DON such as amino acids and humic substances. Thurman

(1986), for example, observed increases in amino acid concentrations associated with spring snow melt. Further investigations of this kind may identify specific DON sources that influence bulk DON concentrations (Neal *et al.*, 2000c).

Table 3.3. Investigations of seasonal variations in DON concentrations

Temporal variation present	
Study area	**Reference**
Chesapeake Bay, USA	McCarthy *et al.*, 1977; Keil and Kirchman, 1991; Bronk *et al.*, 1998
Tomales Bay, USA	Smith *et al.*, 1991
Field studies of subsurface flow	Heathwaite *et al.*, 2000
North Inlet, USA	Lewitus *et al.*, 2000
Streams in the Scottish uplands	Chapman *et al.*, 2001
Rivers draining into the Baltic Sea	Stepanauskas *et al.*, 2002
Temporal variation absent	
Rivers in SE USA	Alberts and Takacs, 1999
Riverine inputs to the Gulf of Riga, Baltic Sea	Laznik *et al.*, 1999
White Mountain streams, USA	Goodale *et al.*, 2000
Appalachian streams, USA	Buffam *et al.*, 2001
Rocky Mountain streams, USA	Kaushal and Lewis, 2003
SE Carolina estuaries, USA	White *et al.*, 2004

Temporal variation was apparent from DOC concentrations in both freshwater and saline samples, even though none had been observed for DON. This indicates that different processes influence the breakdown of DOC and DON in river and estuarine environments (Russell *et al.*, 1998). The high DOC: DON ratio observed in both winters indicates carbon rich DOM and supports this idea, particularly in the saline waters as high concentrations of DOC relative to DON were recorded in these samples (Williams, 1995). Possibly an additional source of DOC enters the river and estuary during these months, or there is more breakdown and removal of DON (Williams *et al.*, 2001). The highest DOC: DON ratio was recorded at Redbridge immediately downstream of the salt marshes, in November and January 2001, suggesting that this area was important for DON removal. The timing of this decoupling between DOC and DON was earlier than expected as it was

prior to spring increases in biological growth and summer low flows which are associated with high rates of decomposition (Russell *et al.*, 1998).

Despite the lack of seasonal variation in DON concentrations in the River Test and estuary, temporal changes were apparent in DOC. Minimum DOC was recorded in the spring (April), suggesting high rates of removal with concentrations accumulating through the summer until reaching a maximum in the autumn. This seasonal increase in DOC throughout the summer has been observed in other studies (Miller, 1999; Goodale *et al.*, 2000; Cauwet, 2002) and has been associated with lower flow conditions (Clair *et al.*, 1996; Stepanauskas *et al.*, 1999b) and higher rates of leaf litter decay (Tipping *et al.*, 1997; Hessen, 1999) as well as the increase in DOM release associated with low bacterial uptake due to low concentrations of inorganic nutrients (Williams, 1995).

A close relationship has been observed throughout this study between DOC and flow. However, during the higher flows of autumn 2002 concentrations of DOC increased with flow. Similar patterns have been associated with diffuse sources of DOC in the River Wear (Neal *et al.*, 2000b) and Mississippi (Benner and Opsahl, 2001). The seasonality in this relationship suggests that these diffuse sources of DOC are particularly important during high flow conditions (Buffam *et al.*, 2001).

Some seasonal variation was apparent in the DOC: DON ratio in freshwater samples with more carbon-rich DOM occurring in April and more nitrogen-rich DOM in the winter. However, this pattern was not consistent between the years or repeated in saline samples. In fact the temporal variation was quite erratic with the saline samples containing markedly more carbon-rich DOM in the winter. Although higher DOC: DON tend to be associated with terrestrially derived DOM (Clair *et al.*, 1996), these higher values were observed in saline samples. It is suggested therefore that the higher DOC: DON from this study were probably associated with the small number of samples considered for each month, which meant that small differences in concentration of DON and DOC resulted in large changes in DOC: DON (Goodale *et al.*, 2000). No covariation was observed between DOC and DON throughout the 18 month study, with the exception of winter 2001, which suggests that the production and utilisation of DON is not strongly coupled with DOC (Wheeler and Kirchman, 1986; Hopkinson *et al.*, 1993).

3.4.3 Nitrogen load entering the Test estuary

The annual DON load was an order of magnitude greater than the ammonium and an order of magnitude less than nitrate load; DON comprises about 10 % of the total dissolved nitrogen load. The relationship between monthly DON load was not in such close agreement with flow as seen for DIN, although the peak load coincided in December 2002 for all dissolved constituents of nitrogen.

The study of total dissolved nitrogen load for the Rivers Test and Itchen between 1974 and 1997 suggest a much lower load compared with this study (Table 3.4)(Hydes and Wright, 1999). Although the study by Hydes and Wright (1999) includes the River Itchen (which also drains into Southampton Water), there was no allowance for the DON load which could account for a ten percent increase in the total load. The reasons for the discrepancy in TDN load between the two studies may be associated with the methods used to calculate the nutrient load. The earlier study calculated the TDN load using conservative mixing relationships and freshwater endmembers from the estuary, and was able to identify a large nitrate sink in the upper estuary which may account for the smaller TDN load from this study.

Despite this, there still appears to have been a marked increase in the TDN loads entering Southampton Water from the River Test since 1998. Some of the variation may be accounted for by variations in river flow, which can vary substantially from year to year. However, closer investigations of annual changes in the mean nitrate concentration show a long term increase in the major component of TDN. The riverine nitrate concentration increased from an average of 342 µM between 1974-1979, to 422 µM between 1990 and 1997 (Hydes and Wright, 1999). This study has shown a further increase in nitrate concentrations to 462 µM between 2001 and 2002. This was an increase of more than 25 % over the last 25 years and appears to be continuing.

The pollutant load of the River Test in 1994 was also calculated by HR Wallingford in their report EX 3253 (1995), given in Shi (2000). The nutrient loads were estimated using water quality concentrations and daily discharge in July 1994 (Russell *et al.*, 1998). The ammonium load has apparently changed very little between 1994 and 2002.

Study area	Year of study	TDN		Nitrate		Ammonium		DON		Reference
		Load	AN load ($\times 10^5$)	Load	AN load ($\times 10^5$)	Load	AN load ($\times 10^3$)	Load	AN load ($\times 10^4$)	
UK										
Southampton Water	1971 - 1973	-	-	220	-	5.2	-	-	-	(Wright, 1980)
Upland catchments	Not known	-	-	-	0.25	-	-	-	1.4 – 2.1	(Edwards *et al.*, 1996)
Great Ouse	1992	-	-	372	-	-	-	-	-	(Rendell *et al.*, 1997)
River Severn	1995	-	1.3	-	1.1	-	1.5	-	2.0	(Russell *et al.*, 1998)
River Avon	1995	-	1.6	-	1.4	-	0.6	-	2.2	(Russell *et al.*, 1998)
River Exe	1995	-	1.6	-	1.3	-	1.4	-	2.4	(Russell *et al.*, 1998)
River Dart	1995	-	2.3	-	1.9	-	1.4	-	3.9	(Russell *et al.*, 1998)
Rivers Test and Itchen	1974 - 1998	131^	-	-	-	-	-	-	-	(Hydes and Wright, 1999)
River Test	1998	-		115	-	2	-	28	-	(Shi, 2000)
River Itchen	1998	-	-	10	-	1	-	51	-	(Shi, 2000)
Severn estuary	1995 – 1996	-	-	1996	1.3	285	1.8	-	-	(Nedwell *et al.*, 2002)
Mersey estuary	1995 – 1996	-	-	2667	7.8	1295	38	-	-	(Nedwell *et al.*, 2002)
Morecambe Bay	1995 – 1996	-	-	210	0.8	75	2.7	-	-	(Nedwell *et al.*, 2002)
Garnock estuary	1995 – 1996	-	-	66	0.9	5	0.6	-	-	(Nedwell *et al.*, 2002)
Tyne estuary	1995 – 1996	-	-	309	1.1	236	8.0	-	-	(Nedwell *et al.*, 2002)
Wear estuary	1995 – 1996	-	-	95	0.8	23	2.0	-	-	(Nedwell *et al.*, 2002)
Tees estuary	1995 – 1996	-	-	226	1.2	100	5.2	-	-	(Nedwell *et al.*, 2002)
Humber estuary	1995 – 1996	-	-	2971	1.5	252	1.3	-	-	(Nedwell *et al.*, 2002)
Wash estuary	1995 – 1996	-	-	426	0.7	69	1.1	-	-	(Nedwell *et al.*, 2002)
Stour estuary	1995 – 1996	-	-	46	0.8	0.3	0.1	-	-	(Nedwell *et al.*, 2002)

Table 3.4. Summary of nutrient loads (Mmol y^{-1}) and area-normalised (AN) (mol km^{-2} y^{-1}) loads for TDN, nitrate, ammonium and DON. Values in brackets indicate total load including terrestrial inputs from sewage treatment works. * Indicates DON and PON and # mean nitrate and nitrite.

Study area	Year of study	TDN		Nitrate		Ammonium		DON		Reference
		Load	AN load ($\times 10^5$)	Load	AN load ($\times 10^5$)	Load	AN load ($\times 10^3$)	Load	AN load ($\times 10^4$)	
Colne estuary	1995 – 1996	-	-	43	1.7	21	8.2	-	-	(Nedwell *et al.*, 2002)
Blackwater estuary	1995 – 1996	-	-	177	1.5	2	0.2	-	-	(Nedwell *et al.*, 2002)
Thames estuary	1995 – 1996	-	-	2306	2.0	483	4.3	-	-	(Nedwell *et al.*, 2002)
Medway estuary	1995 – 1996	-	-	225	1.6	86	6.2	-	-	(Nedwell *et al.*, 2002)
Pegwell estuary	1995 – 1996	-	-	36	1.0	0.4	0.1	-	-	(Nedwell *et al.*, 2002)
Rother estuary	1995 – 1996	-	-	23	0.4	0.4	0.1	-	-	(Nedwell *et al.*, 2002)
Arun estuary	1995 – 1996	-	-	156	2.9	2	0.4	-	-	(Nedwell *et al.*, 2002)
Christchurch Hbr	1995 – 1996	-	-	469	1.7	5	0.2	-	-	(Nedwell *et al.*, 2002)
Plymouth Sound	1995 – 1996	-	-	150	1.1	3	0.2	-	-	(Nedwell *et al.*, 2002)
Southampton Water	1995 – 1996	-	-	210 (228)	1.8 (1.9)	6.1 (54.9)	5.1 (45.9)	-	-	(Nedwell *et al.*, 2002)
River Test, UK	2002	231	1.9	206	1.7	2.1	1.8	23	1.9	This study
Worldwide										
7 rivers, Australia	1995 - 1996	125	1.1 - 4	14 - 28	0.03 – 0.1	7	0 – 2.9	14 - 207	6.4 – 31.4	(Eyre and Pont, 2003)
Global load	-	1.0×10^9	-	2.8×10^8	-	3.6×10^4	-	7.1×10^8	-	(Meybeck, 1982)
European summary	Not known	-	-	0.1 - 25	-	<0.1 – 5.3	-	-	-	(Kempe *et al.*, 1991)
26 rivers, Canada	1983 - 1989	-	-	-	-	-	< 2.9	54	5.9*	(Clair *et al.*, 1996)
Rivers entering Gulf of Riga	1977 -1995	236 - 4807	0.5 – 0.7	11 - 1386	0.2 – 0.5	8-209	-	-	-	(Laznik *et al.*, 1999)
Lemon Bay, USA	1995	9.3	0.6	-	-	-	-	-	-	(Tomasko *et al.*, 2001)
34 estuaries, USA	Not known		0.7 - 35							(Castro *et al.*, 2003)

Table 3.4 Continued ^ indicates DIN only.

A marked increase in the nitrate load was observed over the 8 years, which is in agreement with the measured increases in nitrate concentrations. An estimate was also made in this report of the organic nitrogen load, although it is not known whether this was the dissolved, particulate or total organic nitrogen load. The organic nitrogen load in 1994 was calculated as 29 Mmol y^{-1} which was slightly higher than the DON load of 23 Mmol y^{-1} estimated in 2002 from this study. Without further information about the form of organic nitrogen it is not possible to comment on changes to the nutrient load between the two studies.

The area-normalised total dissolved nitrogen load of the River Test was in good agreement with other UK river catchments and was only exceeded by the River Dart. It has been suggested that the River Dart has a larger load than many other UK catchments, as large amounts of fertiliser are applied to sustain high grazing and maximise silage production (Russell *et al.*, 1998). The importance of using area-normalised loads to draw direct comparisons between catchments independent of their size was highlighted from the study of the Rivers Avon (which flows into the River Severn) and Exe which had identical area-normalised loads despite the catchment area of the Avon being more than four times that of the Exe (Russell *et al.*, 1998).

Investigations into the constituents of the dissolved nitrogen load have been far more extensive for DIN than DON. Summaries of nitrate loads from UK estuaries show annual loads between 0.4 and 7.8 x 10^5 mol km^{-2} yr^{-1} (Russell *et al.*, 1998; Nedwell *et al.*, 2002). The loads from UK upland catchments (Edwards *et al.*, 1996) and rivers draining into the Gulf of Riga (Baltic Sea) appear to be much lower (Laznik *et al.*, 1999). This study is in good agreement with other UK studies of nitrate loads and tends to be towards the upper range of estimated loads (Nedwell *et al.*, 2002). This is probably associated with the fact that the River Test is fed from aquifers containing stores of water that have been held underground for many years. Recent changes in legislation restricting the use of fertilisers in nitrate sensitive areas are likely not to impact this catchment for many years due to the long storage time of water entering the River Test from the aquifer.

Extensive studies have been carried out on the ammonium loads entering UK estuaries from riverine sources. The highest loads were observed entering the heavily industrialised Mersey estuary, probably associated with the high population density and therefore

discharge of sewage effluent inputs to the catchment (Nedwell *et al.*, 2002). The ammonium load calculated for the Test estuary from this study is relatively low compared to other UK estuaries (Table 3.4). This riverine load does not account for effluent inputs entering the estuary below the tidal limit gauging station. The significance of this input into Southampton Water is highlighted in the study by Nedwell *et al.* (2002) as only 3 % of the total (riverine plus sewage effluent) load is derived from riverine sources. As neither study by Wright (1980) or Nedwell *et al.* (2002) differentiates between the loads entering Southampton Water from the Test and Itchen rivers it is difficult to interpolate the input solely from the River Test. However, since the ammonium loads in those studies are two to three fold larger than seen in this study, it does suggest that the STW inputs from the Itchen are either larger, or the total load entering Southampton Water in 2002 decreased relative to the previous studies (Wright, 1980; Nedwell *et al.*, 2002). The River Test appears to have an average input of area-normalised ammonium load entering the estuary comparable to other UK river systems.

There have been relatively few attempts to compare estimates of DIN load to DON in rivers on a worldwide basis. At present the author was not aware of any other estimates of DON loads or area-normalised loads from UK rivers. However, there were studies investigating area-normalised DON loads in UK rivers (Edwards *et al.*, 1996; Russell *et al.*, 1998). The River Test appears to have a low annual load in comparison with other worldwide studies (Clair *et al.*, 1996; Eyre and Pont, 2003). The annual area-normalised load was in good agreement with most lowland UK studies (Russell *et al.*, 1998).

3.5 CHAPTER SUMMARY

This report represents the first estimate of riverine DON load and area-normalised load from a UK river system and is in good agreement with other studies of DON worldwide. DON is an important component of both the total dissolved nitrogen composition and nutrient load entering the Test estuary. DON accounts for the second largest fraction of dissolved nitrogen and comprises up to 26 % of total nitrogen in saline samples collected in the upper estuary. The higher concentrations were measured in saline compared to freshwater samples although no correlation was observed between DON and salinity.

Clearly identifiable temporal variations were not apparent over the 18 month survey in either the DON concentration or composition of TDN, which was in contrast to the clear

seasonal variation in DOC concentrations. This observation suggested a decoupling of the carbon and nitrogen in dissolved organic matter, which was particularly evident from the carbon rich DOM measurements in the winter months.

DON measured in the River Test and estuary was found to be an important fraction of the total nitrogen composition. DON represents the second largest fraction of nitrogen and accounts for up to 26 % of the total nitrogen entering the estuary. Concentrations of DON were higher in the estuary than in the River Test, although there was no clear relationship between DON and salinity. The DON load entering the Test estuary was an order of magnitude higher than ammonium, and an order of magnitude less than nitrate. The monthly DON load showed different temporal variation to the DIN species, suggesting a weaker relationship between DON concentration and flow. Compared to other previous studies of DIN in the Test river there appears to be long term an increase in nitrate concentration, which cannot be accounted for by inter-annual fluctuations in the river flow. The riverine ammonium load was lower than many other UK and worldwide studies, although no account was made for estuarine sewage effluent inputs.

4 CHAPTER FOUR. SPATIAL VARIATIONS IN INORGANIC AND ORGANIC FORMS OF NITROGEN AND CARBON IN THE RIVER TEST – ESTUARY

4.1 INTRODUCTION

The waters of the River Test are characterised by a high water clarity and low sediment load, which can be attributed to filtering of water by the chalk-bed aquifer. These properties are exploited in the upper catchment for washing bank notes as well as cress farming (Environment Agency, 2002). The area is famous for fly-fishing and has become an EC designated freshwater salmonid fishery. Fishing is an important industry as well as sport on the river with numerous fish farms scattered along its length, as well as fish passes and counters located downstream of Romsey (Environment Agency, 1999).

There are additional demands on the resources of the River Test as the lower catchment is urbanised and therefore requires abstraction of large volumes of water as well as effluent disposal via both public and private sewage treatment works (STW) (Figure 4.1). The largest of these public STW are located at Andover, Romsey and Millbrook the latter being on the upper Test estuary (Wright, 1980; Hydes, 2000). These point effluent sources are regulated by the Environment Agency to ensure compliance with the Urban Waste Water Treatment Directive (UWWT). This is particularly important as the area south of Andover is considered at risk of becoming eutrophic (Environment Agency, 1999). Frequent chemical monitoring asserts whether effluents entering the river remain within consented limits. However, monitoring of DON and DOC concentrations downstream of these effluents input is not carried out.

The intertidal reaches of the lower Test include salt marshes that are protected as a nature reserve (Appendix H). Salt marshes are known to influence nutrient concentrations and specifically act as a nitrogen store (Stepanauskas *et al.*, 1999b). Few studies have been carried out on the impact of salt marshes on DOM and most of these have focused on DOC rather than DON (Nedwell *et al.*, 1999).

The aim of this part of the study was to assess spatial changes in DON and DOC along the Test river / estuary continuum in order to quantify the magnitude of point source inputs and the influence of salt marshes on nutrient concentrations in the tidal reaches.

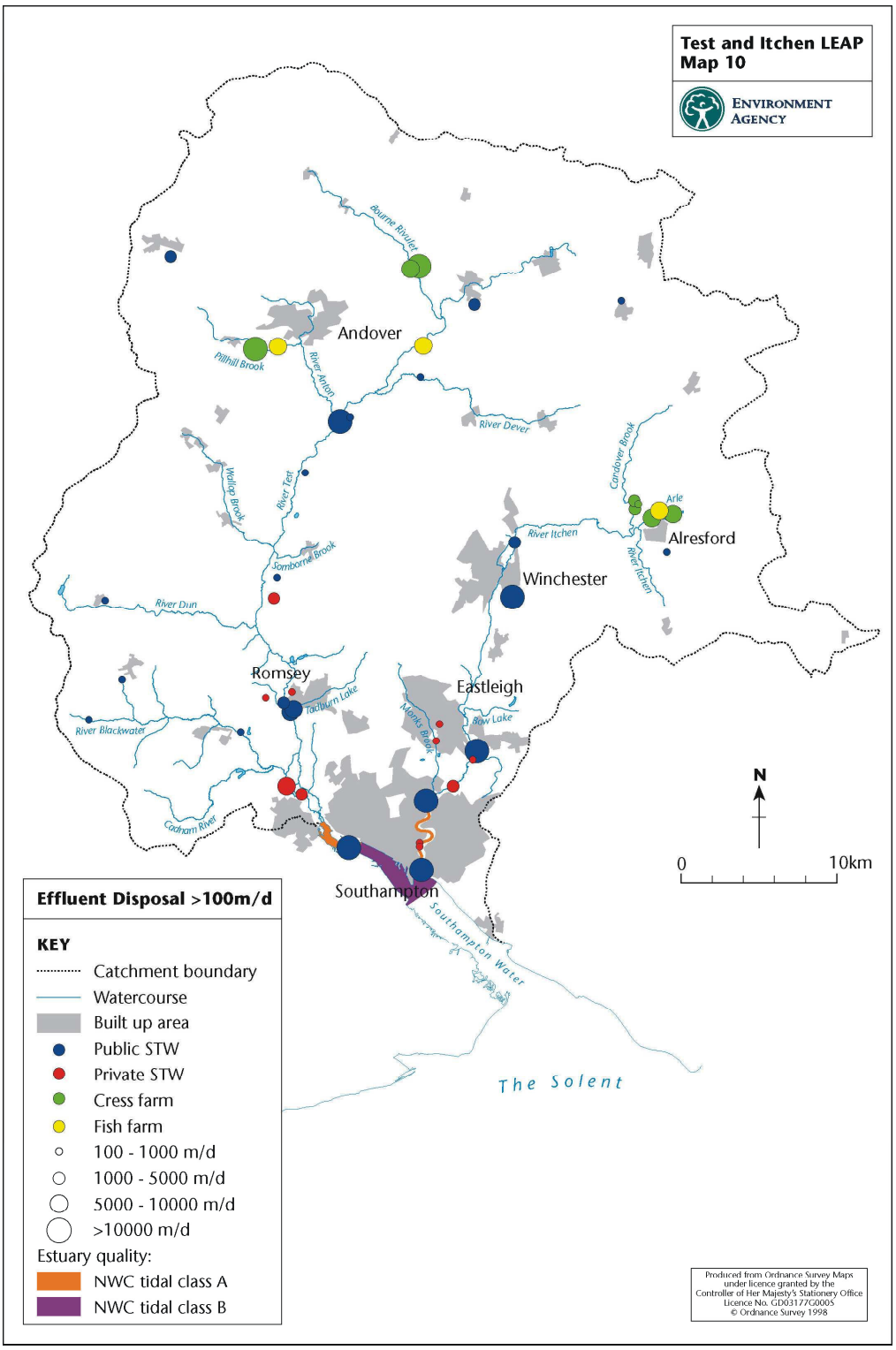


Figure 4.1. Effluent disposal in the Test catchment, Hampshire (Environment Agency, 1999). STW means sewage treatment work and NWC is the National Water Council.

4.2 METHODOLOGY

A sampling regime was designed to determine the influence of point source inputs on nutrient concentrations in certain reaches of the river. An extensive spatial sampling programme was established to characterise fully the variations in nutrient concentrations along the mid to lower river and head of the estuary. This sampling area was located between Mottisfont upstream of Romsey, to Redbridge railway station on the upper Test estuary. Samples were collected from several parallel channels wherever the River Test was anastomosed (divided into several channels).

In the lower River Test and upper estuary, restricted access limited the number of sampling locations. From June 2002 the estuarine site at Redbridge Park became accessible to the public and this sampling site was included in later surveys. There are numerous channels of the River Test entering the estuary through the salt marshes at Totton with the freshwater flow and tidal inundation being greater in some channels than others. For example, on the eastern channel of the lower Test the limit of saline inundation was around 1.6 km upstream of Redbridge, which was 0.3 km higher than on the western channel. Sampling of sites within the area of tidal inundation (sites 6 and 6a) was only possible on the eastern channel, since limited access prevented sampling of a range of tidal conditions on the western channel between site 3 and Redbridge. Nutrient samples were analysed using a standard suite of analytical procedures (see §2.3).

4.3 RESULTS

4.3.1 Nutrient observations

Nutrient data Nitrate, ammonium and chlorophyll a concentrations were determined at all sites and their positions indicated on figures relative to their distance upstream from Redbridge. To assess only spatial variations in the nutrient concentrations, all freshwater samples (salinity values < 0.1) are presented in Figure 4.2. The clustering of data indicates samples collected on parallel channels, e.g. sites 4 and 5 at 1.5 km, sites 7 and 8 at 3.5 km and sites 12, 13 and 14 between 15.3 and 15.4 km upstream of Redbridge.

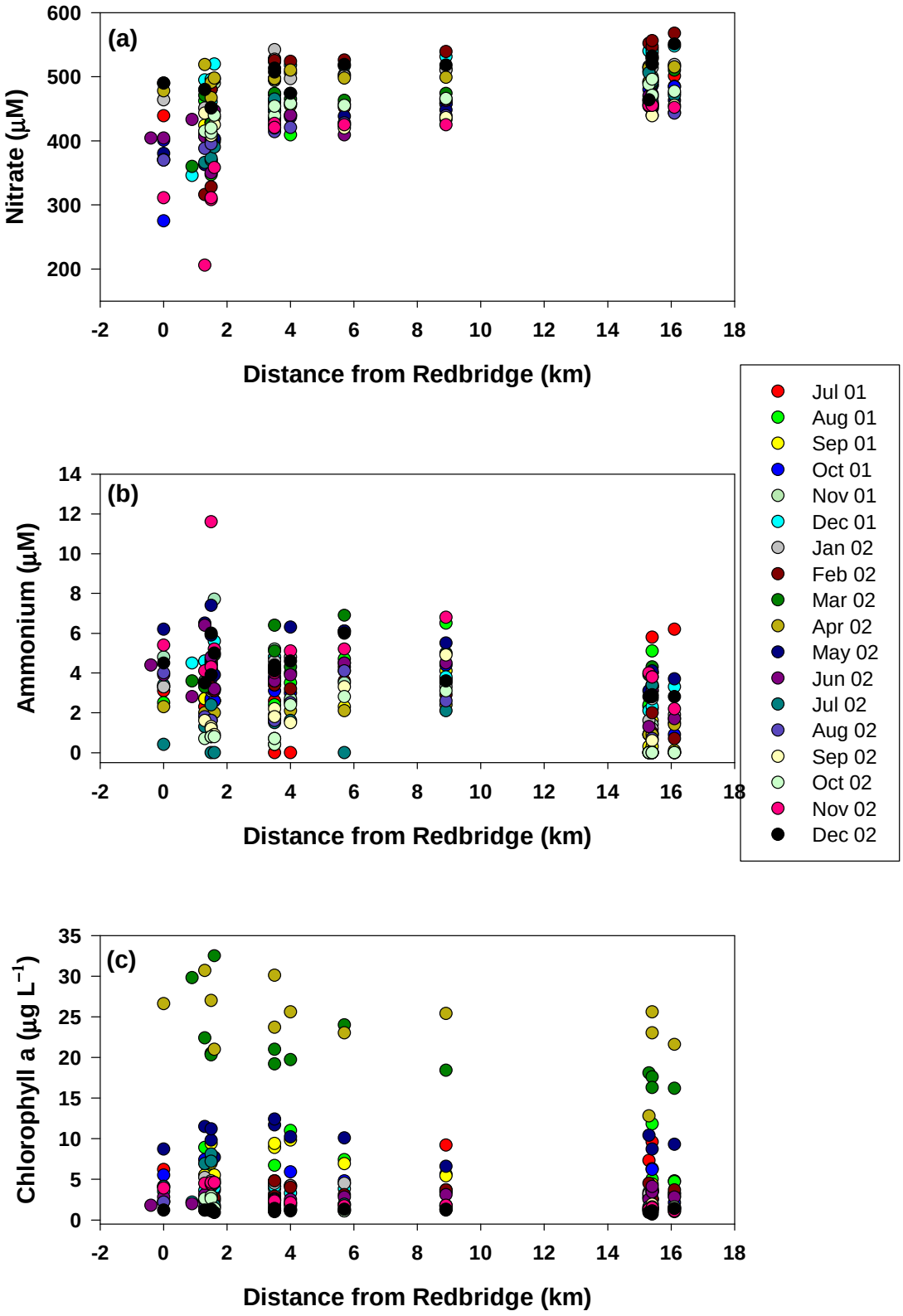


Figure 4.2.Concentration of (a) nitrate, (b) ammonium and (c) chlorophyll a at all freshwater sites (with salinity values < 0.1). Ammonium measurements < limit of detection (0.3 µM) are assumed to be zero.

Nitrate concentrations decreased gradually from the upper to the lower reaches of the study area (Figure 4.2a), despite a slight increase at 3.5 km upstream from Redbridge (sites 7 and 8). In the upper reaches of the study area between 400 and 500 µM nitrate was measured, whereas, in the lower reaches a much wider range of concentrations were observed with < 300 µM being measured in October 2001 and November 2002.

Ammonium concentrations were consistent throughout the River Test with the majority of measured values between 0.4 µM (limit of detection) and 7 µM (Figure 4.2b). In the lower Test a wider range of ammonium values was recorded up to a maximum of 6 µM in November 2002. The ammonium concentration measured at site 11 (9 km upstream of Redbridge) was notably always greater than 2 µM.

No clear spatial variation was apparent in the chlorophyll a concentrations (Figure 4.2c). The majority of sites had concentrations ranging from 1 to 13 µg l^{-1}. Concentrations in excess of 15 µg l^{-1} were recorded at most sites in February and March 2002 which were markedly higher than the concentrations measured in other months. The highest chlorophyll values were measured between 1.3 and 3.5 km upstream of Redbridge.

Small variations in the nutrient concentrations were apparent between sites on parallel channels of the River Test (Figure 4.2), therefore in the remainder of this section data from sites 5, 8, 12 and 14 are omitted in favour of sites 4, 7 and 13, which had the highest river flow. No further data will be presented from site 3 as similar concentrations were measured at site 4, which is located only 200 m upstream (Figure 2.1).

Nutrient changes along the river - estuary continuum Monthly sampling between July 2001 and December 2002 was used to characterise spatially the nutrient concentrations throughout the river to estuary continuum. The data presented in Figure 4.3 to Figure 4.5 show the spatial distribution of nutrient concentrations focussing on main channel freshwater sites (sites 2, 4, 7, 9, 10, 11, 13 and 15) in samples with salinity values < 0.1. Where parallel channels were located, the site with the highest river flow was selected to represent the main channel site. Eighteen months of nutrient concentrations at each sampling site have been summarised using box and whisker plots with the number in brackets above the x-axis indicating the number of monthly values included for each site.

Median nitrate concentrations gradually decreased downstream from site 15 to site 4 (1.5 km north of Redbridge), with a range of 100 µM measured at each sample site (Figure 4.3a). A marked decline in the median concentration was observed at site 4 and 2 relative to the mid and upper reaches of the study area, and a larger range of concentrations was measured than further upstream. The widest range of concentrations was recorded at site 7 where nitrate concentrations <310 µM were measured in September and October 2002.

Ammonium concentrations measured in the River Test were consistently <7.5 µM (Figure 4.3b). When a concentration less than the limit of detection (0.4 µM) was recorded, this was represented as zero. Although there was no consistent increase or decrease in concentrations throughout the study area, a similar range of concentrations was measured at all sites. The range of median ammonium concentration was 0.4 – 7.4 µM and fluctuated between sample sites along the length of the river.

An increase in median dissolved organic nitrogen (DON) concentration was observed from 26 to 42 µM downstream, although there was a decrease at the most downstream point, site 2 at Redbridge (Figure 4.3c). Two exceptions to this trend were sites 7 and 13, although data from site 7 do not represent the full 18 month survey as only two data values were collected. A marked increase in DON was observed at site 13 relative to site 15, despite being only 600 m downstream. This site was located in an area of intense fish farming and data from a parallel channel (site 12) will be discussed further (§4.3.3).

A wide range of dissolved organic carbon (DOC) concentrations was observed, particularly in the lower Test at site 2 (Figure 4.4a). A gradual downstream increase in median DOC (from 100 to 175 µM) was observed, similar to that seen for DON (Figure 4.3c), as well as elevated median concentrations at site 13. Median DOC concentrations at site 2 were only slightly lower than at site 4.

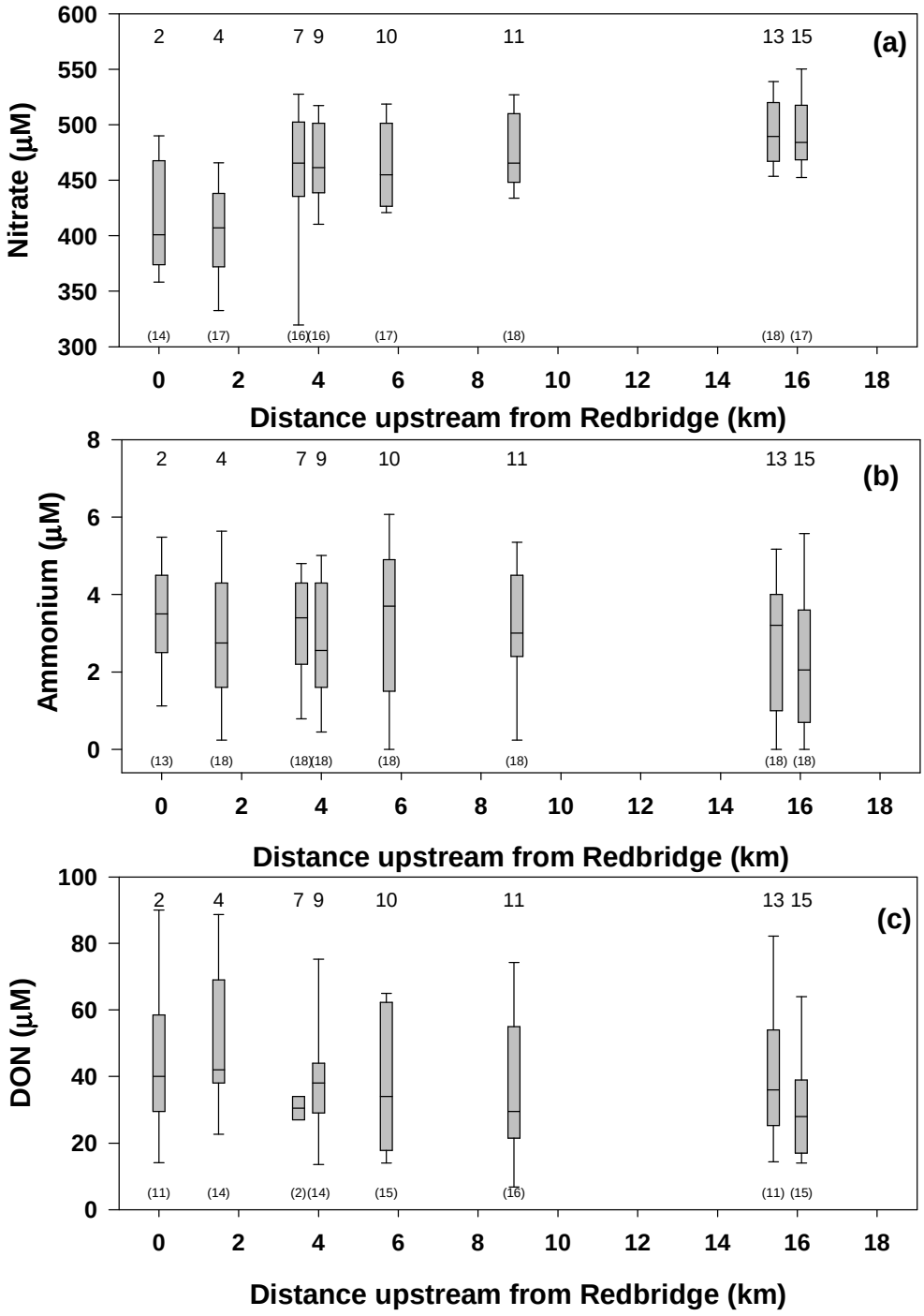


Figure 4.3. Spatial variations in (a) nitrate, (b) ammonium and (c) DON measured in freshwater samples from sites 2, 4, 7, 9, 10, 11, 13 and 15 (site number indicated at top of panel (a)). The upper box boundary represents 75th percentile and lower, 25th. The whiskers above and below the box indicate 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. The number in brackets above the x-axis represents the number of monthly values included for each site. Note non zero intercept for y axis on panel (a).

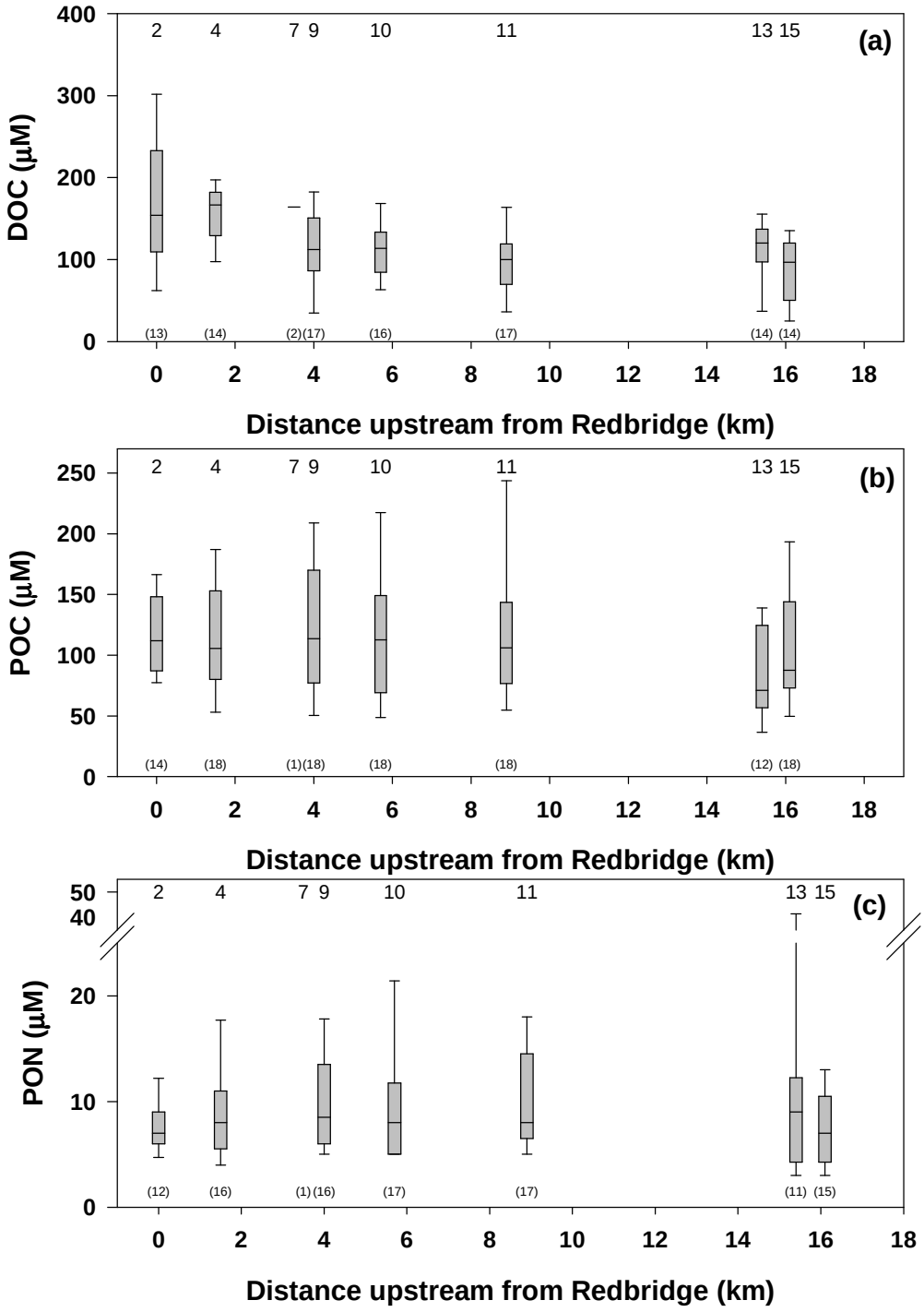


Figure 4.4. Spatial variations in (a) DOC, (b) POC and (c) PON measured in freshwater samples from sites 2, 4, 7, 9, 10, 11, 13 and 15 (site number indicated at top of panel (a)). The upper box boundary represents 75th percentile and lower, 25th. The whiskers above and below the box indicate 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. The number in brackets above the x-axis represents the number of monthly values included for each site.

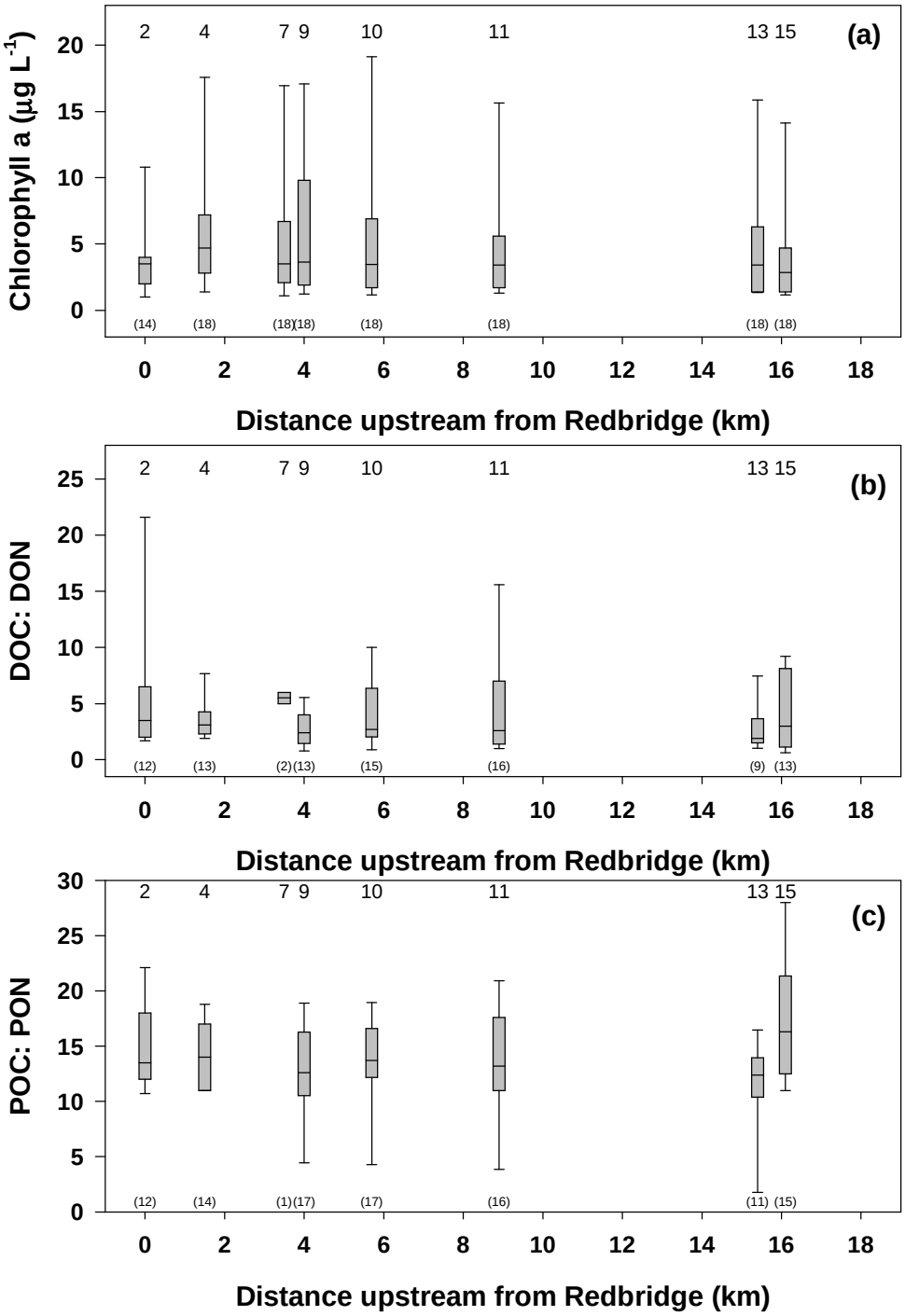


Figure 4.5. Spatial variations in (a) chlorophyll a, (b) POC: PON and (c) DOC: DON measured in freshwater samples from sites 2, 4, 7, 9, 10, 11, 13 and 15 (site number indicated at top of panel (a)). The upper box boundary represents 75th percentile and lower, 25th. The whiskers above and below the box indicate 10th and 90th percentiles. The line within the box represents the median. Computations of whisker percentiles and median require > 5 data points. The number in brackets above the x-axis represents the number of monthly values included for each site.

Similar concentrations of particulate organic carbon (POC) and DOC were measured during the 18 month survey period (Figure 4.4b). Median concentrations of POC up to 10 km upstream of Redbridge were all *ca.* 115 µM. Lower median POC of 75 and 90 µM were recorded at sites 13 and 15 respectively. The lowest range of concentrations was measured at Redbridge (site 2).

Concentrations of particulate organic nitrogen (PON) in the River Test and estuary were much lower than the POC concentrations (Figure 4.4c). The median PON concentration in the freshwater samples was *ca.* 8 µM. The spatial variation in PON was similar to POC with the marginally highest concentrations being measured in the mid-reaches of the study area. The largest range of concentrations was recorded at site 13. This was particularly due to high measurements of PON in October and December 2002.

Median chlorophyll a concentration gradually increased downstream from 3 to 4 $\mu g l^{-1}$ (Figure 4.5a). Concentrations at site 4 were slightly elevated relative to the other sites. The median in the box and whisker plots lay closer to the 10^{th} than the 90^{th} percentiles, suggesting a skew in the data, possibly due to seasonal variations.

The median ratio of DOC: DON was *ca.* 2.5 in the mid-reaches of the study area but increased very slightly at the upper (site 15) and lower limits (site 2). There was a high degree of temporal variation in the freshwater samples indicated by the elevated 90^{th} percentile. This was particularly pronounced at sites 2 and 11 (Figure 4.5b). The narrowest range of DOC: DON was observed at site 7, although only two data values were available at this site.

The ratio of POC: PON was similar to DOC: DON in that the median concentrations were highest at sites 2 and 15 and lowest in the mid-reaches (Figure 4.5c). The 90^{th} percentiles were also highest at sites 2 and 15. The median ratio in the mid reaches was between 13 and 14, although the low 10^{th} percentile at these sites indicated that lower ratios were observed during the 18 month survey.

Nutrient concentrations at the source of the River Test Spatial sampling along the 16.5 km transect from Kimbridge (site 15) on the River Test to the estuary did not encompass the entire fluvial system from the river source. Therefore water samples were

collected to characterise concentrations at the source of the River Test on one occasion in February 2003. The location of the source is near the village of Ashe near Overton (Grid reference SU 532 498), however the precise location of the spring, which rises from upper chalk, can vary depending on the groundwater levels (Environment Agency, 1999). Triplicate water samples were collected and analysed for DIN, DON and DOC, POC and PON and mean concentrations are presented in Table 4.1.

Table 4.1. Nutrient concentrations and other variables at the source of the River Test, 4th February 2003. Nitrate errors are one standard deviation of triplicate measurements, TDN and DOC were based on four replicates, duplicate filters were measured for particulate analyses. Measurement units are µM when not specified.

Nitrate	600 ± 2	**DOC**	163 ± 3	**Chlorophyll a (µg l^{-1})**	0.6 ± 0.1
Ammonium	< 0.7	**POC**	12 ± 2	**Temperature (^{o}C)**	8.6
DON	0 ± 12	**PON**	2 ± 1	**Conductivity (µS cm^{-1})**	573

Mean nitrate concentration was higher at the source than the maximum nitrate measured throughout the river during winter 2001 and 2002, which was 568 µM in February 2002. DOC concentration in the spring sample was also higher than the mean DOC concentration of 104 µM measured at site 15 in winter 2001 and 2002. Markedly lower concentrations of POC were recorded at the source of the river Test than site 15 of 137 µM, whereas the PON was within a similar range. Chlorophyll a concentrations were markedly lower than those measured downstream. The water temperature (8.6 ^{o}C) and conductivity (573 µS cm^{-1}) of the stream rising from the chalk aquifer in winter 2003 were both higher than measurements taken at site 15 (at the upper limit of the study area) the previous winter.

4.3.2 Total nitrogen and total organic carbon composition

The percentage composition of TN (comprising PON, DON nitrate and ammonium) and TOC (comprising of DOC and POC) at 8 main saline and freshwater sites (sites, 17, 16, 6, 9, 10, 11, 13 and 15) is presented on a monthly basis in Figure 4.6 to Figure 4.9. These are the same sites as referred to in §4.3.1, although site 6a is omitted due to limited DON, DOC and particulate data for this site.

The composition of TN is presented in Figure 4.6 and Figure 4.7. Nitrate dominates TN at all sites in this aquifer fed system, comprising 80 – 90 % of TN. However, a lower proportion of the TN comprised nitrate in the saline samples. The smallest percentage of

nitrate was measured at a salinity of 3.9 (site 16) in June 2002 at site 16, when only 48 % of the TN at this site comprised nitrate (Figure 4.7). DON was the second largest component of TN and the highest percentages of DON were recorded in the saline samples, measuring 39 % and 28 % in September 2001 and October 2002 respectively. The two highest tides during the sampling survey occurred these sampling days. The range of TN as DON was between 0 and 50 %, although the mean DON composition was closer to 10 %.

The smallest fraction of TN was composed of ammonium, often only 1 % (Figure 4.7). Similarities were observed between DON and ammonium composition, as they both comprised a higher proportion of TN in the saline compared to the freshwater samples. No obvious spatial variation in the proportion of ammonium was apparent. Particulate organic nitrogen comprised the second smallest fraction of the total nitrogen measured in the freshwater and saline samples. There was little spatial variation throughout the continuum with PON comprising only a marginally higher proportion of TN in the saline samples in September of both years. The mean proportion of PON in the samples was 3 %.

The River Test to estuary continuum was established as a nitrate dominated system with fractions of ammonium and DON being a larger component of TN in the saline samples. Apart from these contrasts between saline and freshwater samples, there was little spatial variation in the total nitrogen composition throughout the river to estuary continuum.

The composition of total organic carbon is presented in Figure 4.8 and Figure 4.9. Some spatial variation was also apparent, although the seasonal variation discussed in Chapter 3 appears to have more influence on the TOC composition.

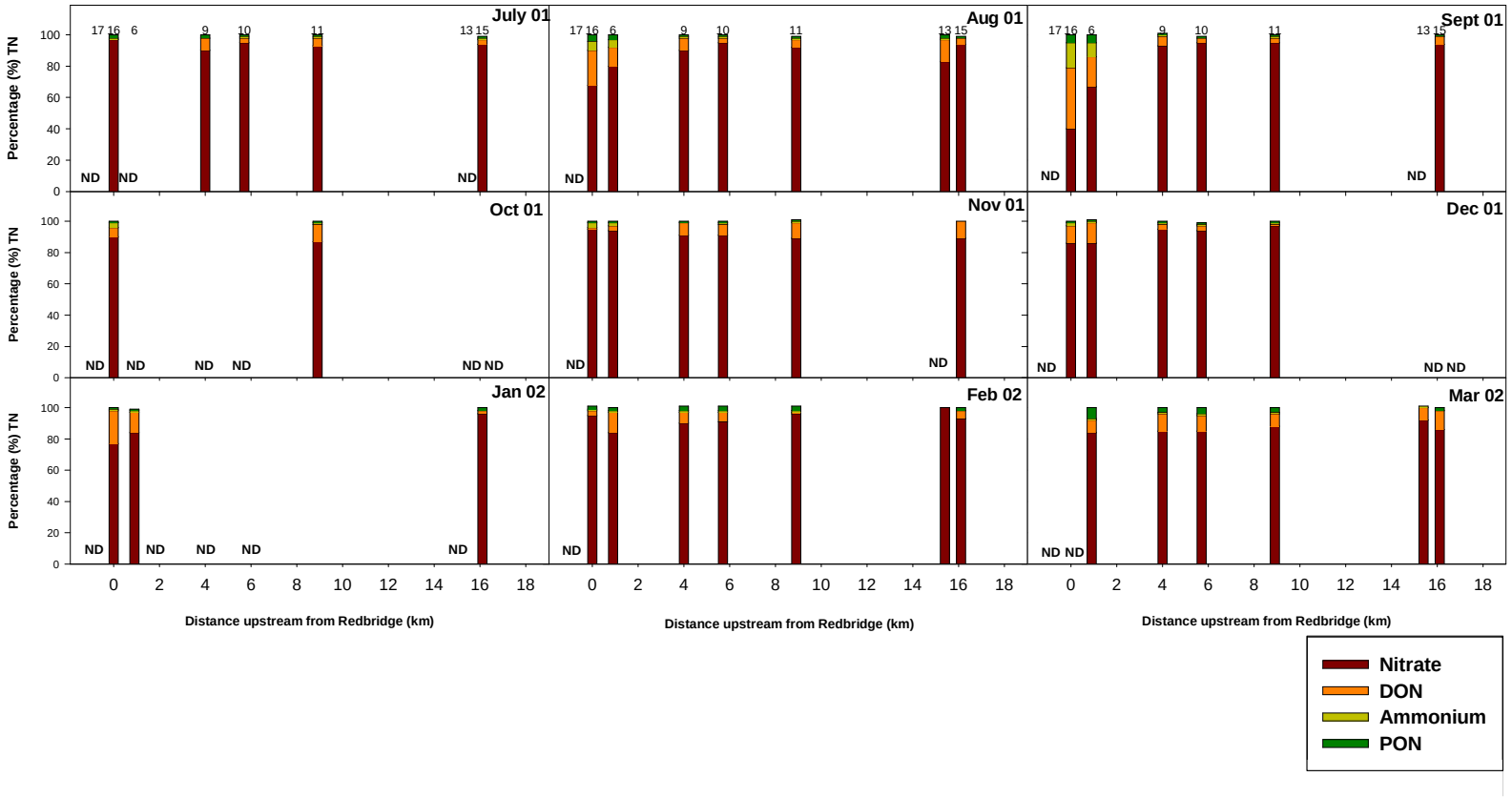


Figure 4.6. Spatial variation in percentage total nitrogen (TN) composition in the River Test and estuary (July 2001 – March 2002) at sites 17, 16, 6, 9, 10, 11, 13 and 15. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

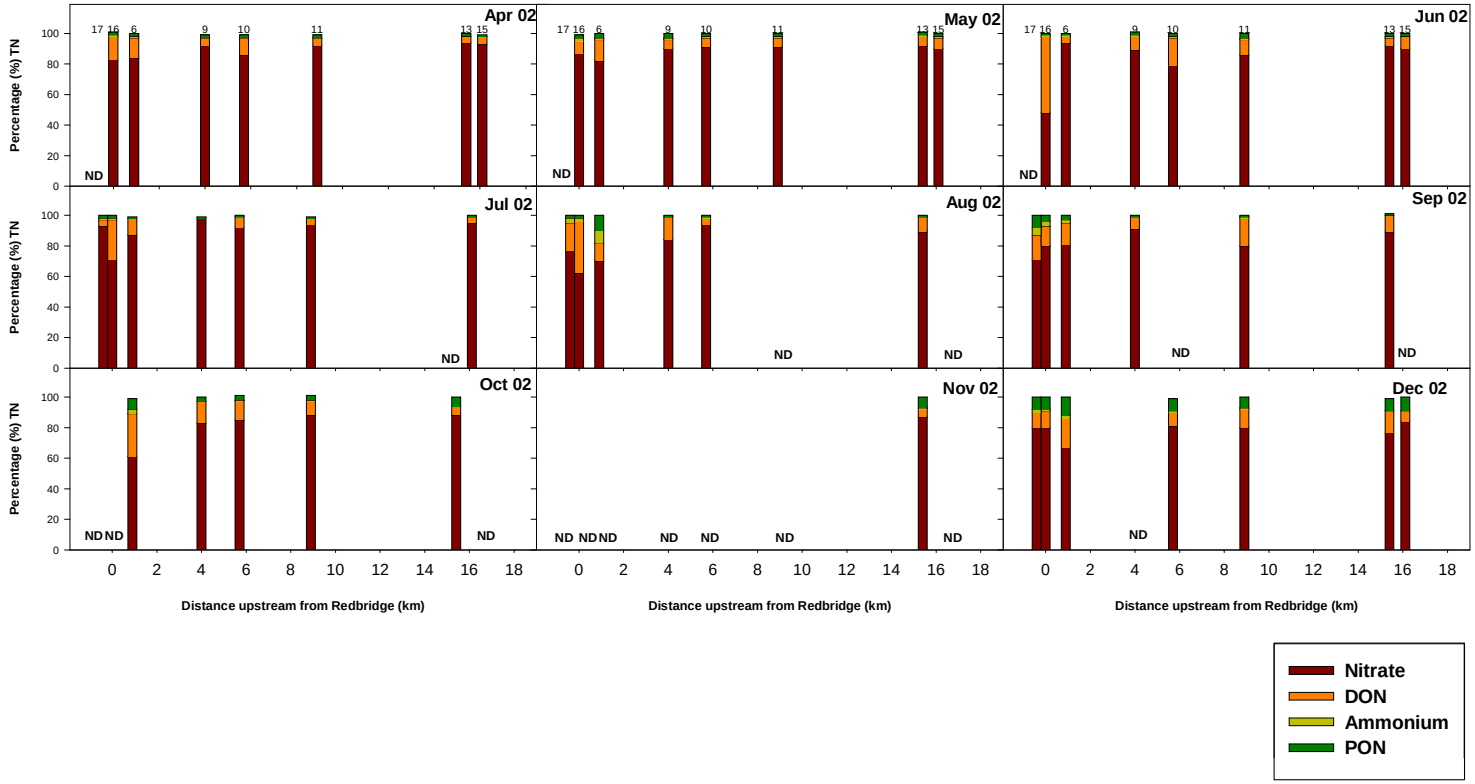


Figure 4.7 Spatial variation in percentage total nitrogen (TN) composition in the River Test and estuary (April – December 2002) at sites 17, 16, 6, 9, 10, 11, 13 and 15. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

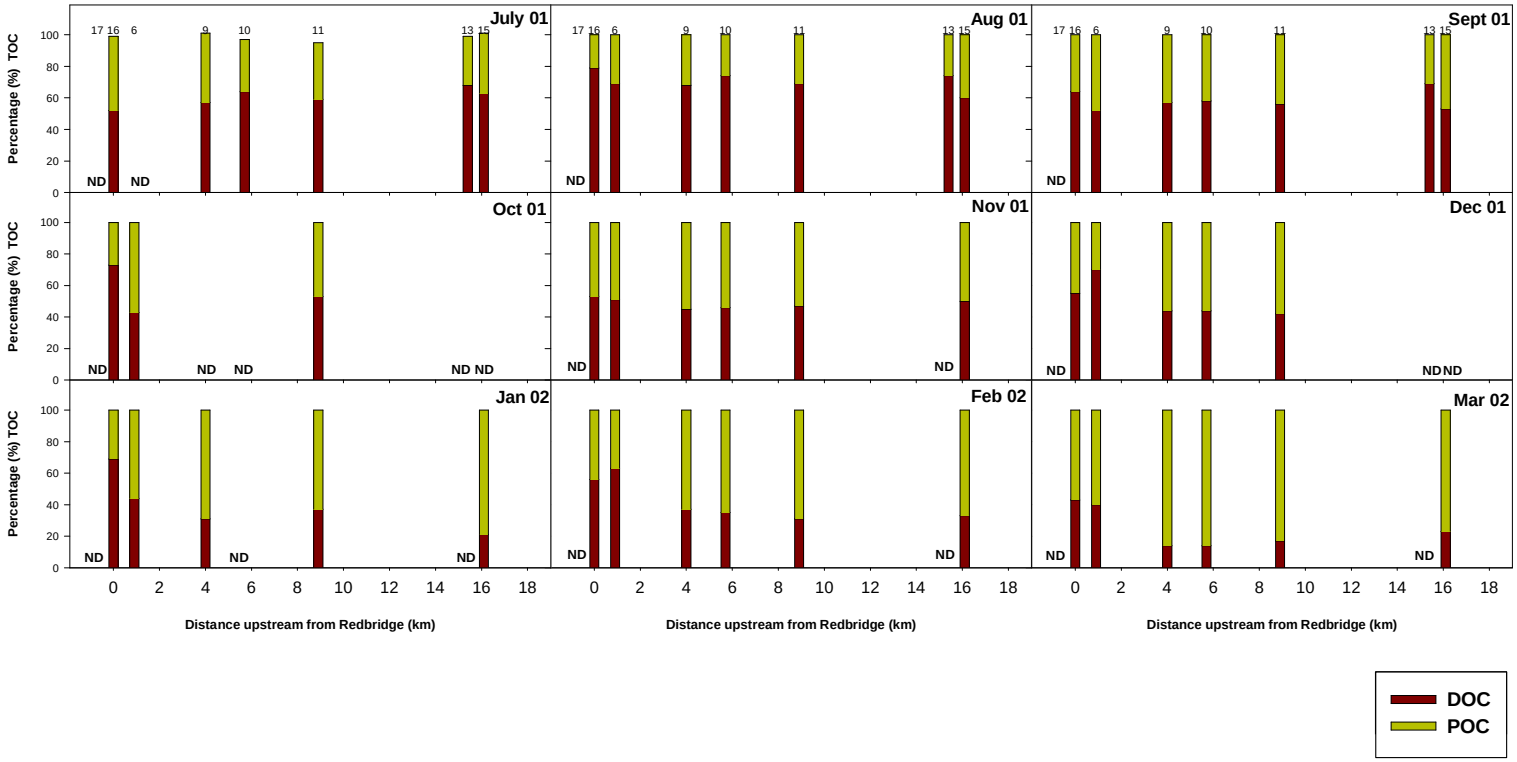


Figure 4.8. Spatial variation in percentage total organic carbon (TOC) composition in the River Test and estuary (July 2001 – March 2002) at sites 17, 16, 6, 9, 10, 11, 13 and 15. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

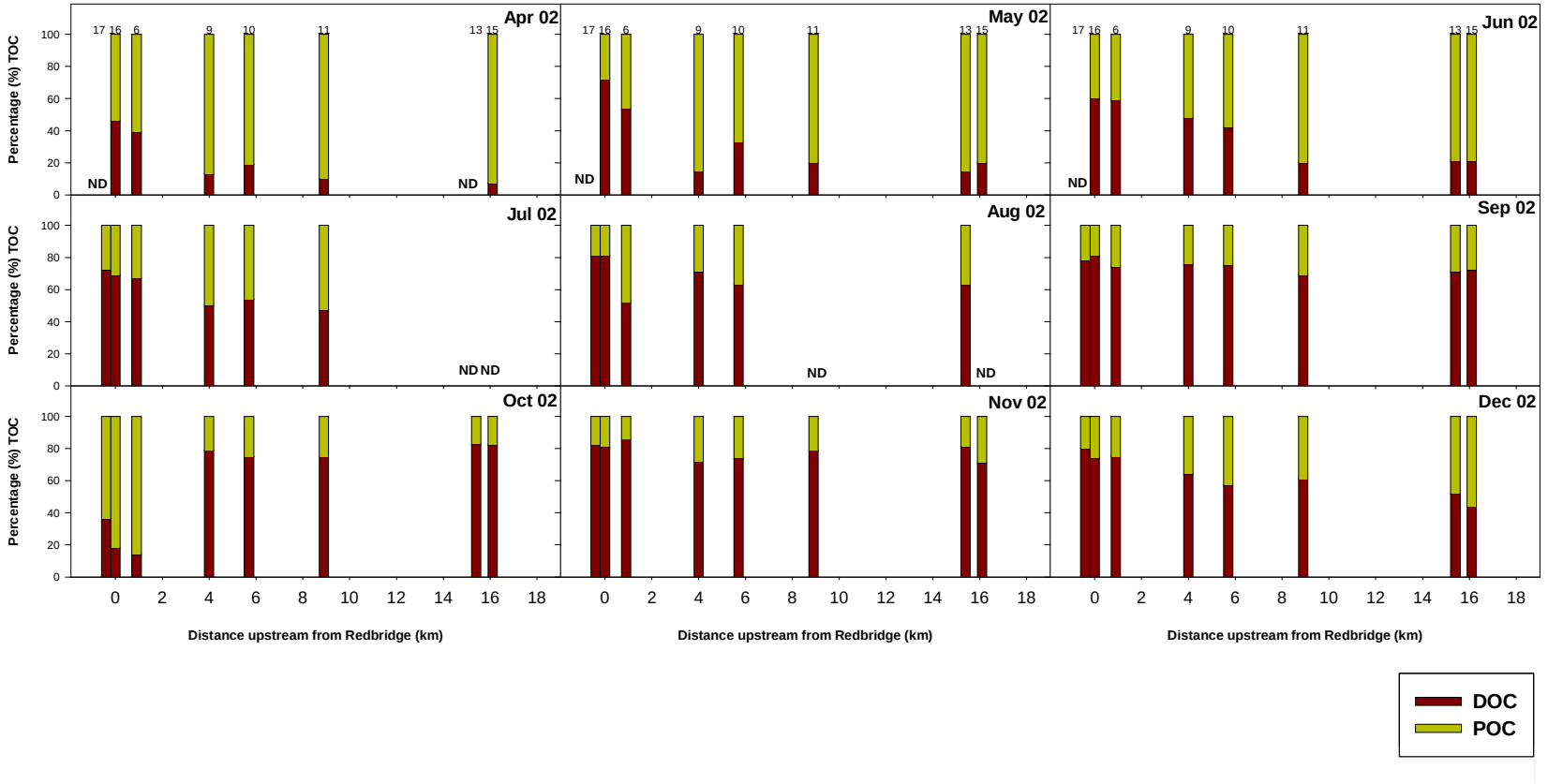


Figure 4.9 Spatial variation in percentage total organic carbon (TOC) composition in the River Test and estuary (April – December 2002) at sites 17, 16, 6, 9, 10, 11, 13 and 15. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

On most dates a gradual transition was observed from POC being the dominant fraction of TOC in the upper reaches of the study area, to DOC dominating in the saline samples. This was particularly prominent in the latter half of 2002 with the exception of October 2002 (Figure 4.9). The highest tide of the sampling period occurred in October 2002, when the particulate organic carbon was the dominant fraction of TOC in the lower part of the study area. Slightly higher percentages of DOC were recorded at site 13 between July and September 2001 compared to the rest of the freshwater samples (Figure 4.8); this pattern was repeated in November and December 2002. In spring and summer 2002 an increase in the proportion of DOC was observed at site 10 relative to site 11. This may be because site 10 is located downstream of the sewage treatment works at Romsey.

4.3.3 Impacts of nutrient sources from (i) a fish farm, (ii) a sewage treatment works and (iii) a salt marsh on the River Test

Some spatial variation in nutrient concentrations was observed throughout the riverine system. Several potential freshwater nutrient point sources were identified along the section of river surveyed including a fish farm at Kimbridge, a sewage outfall at Romsey, and salt marshes between Redbridge and Testwood. Locating sampling sites immediately upstream and downstream of each potential nutrient point source allowed an assessment of the impact of these inputs on the nutrient concentrations of the river. Some results of this work have been published in Homewood *et al.* (2004) (Appendix K)

Kimbridge fish farm The River Test is renowned for its high quality trout fisheries and numerous fish farms are located within the study area, specifically north of Romsey at Kimbridge, at Broadlands Lake and in the Testwood area (Appendices H and L.). The Test River was sampled both immediately downstream of the effluent outfall from the trout fishery at Kimbridge (site 12) and 0.8 km upstream at Mottisfont (site 15). At Kimbridge the river is divided into three channels (Appendix L). The western channel flow (site 13) was considered to be the highest and therefore has been used in previous figures to represent the main channel. The eastern channel (site 14) was the smallest flow and drained disused fish runs, which have subsequently become unused fishing lakes. The middle channel (site 12) flowed around the fish farm and received effluent inputs directly draining from the fish pens. It was anticipated that the point source effluents derived from

fish food, waste products and pest control drugs (Wu, 1995) would contribute ammonium, DON and DOC to the system.

The eighteen-month survey from July 2001 to December 2002 was carried out at locations above and below the fish farm outfall and concentrations of nitrate, ammonium, DON and DOC were measured. No POC / PON analysis was carried out on samples from site 12, therefore only comparisons of the dissolved nutrients are presented.

Nitrate concentrations at sites 12 and 15 are presented in Figure 4.10a for the locations below and above the fish farm effluent outfall. The change in nitrate concentration between the two sites is presented in Figure 4.10b with zero indicating no change in concentrations downstream, positive values indicating an increase downstream between sites 15 and 12, and negative values indicating a decrease. No consistent increase or decrease in nitrate concentrations downstream of the fish farm at Kimbridge was observed. This was confirmed by a one sample t-test of the data (p-value 0.27) that showed that there was no statistically significant difference (at the 95 % confidence level) between the nitrate concentrations at the two sites (Table 4.2). The change in nitrate concentrations in December 2002 appeared anomalous although this may be related to a dilution effect from the very high flow conditions at the time of sampling.

Ammonium concentrations measured at sites 12 and 15 varied during the period of the survey (Figure 4.10c). The concentrations in late summer / early autumn of both years were very low, at points below the detection limit of the analytical technique. This meant that the change in ammonium concentrations downstream was often small or on some occasions no change was recorded. This was apparent from Figure 4.10d, from which it was observed that there was no consistent downstream change in ammonium concentrations. The p-value of 0.74 from the one sample t-test confirmed no statistically significant difference between ammonium concentrations at the two sites (Table 4.2).

Concentrations of DON measured at sites 12 and 15 were generally less than 50 µM, although concentrations in excess of 100 µM were recorded on four occasions (Figure 4.11a). The changes in DON between sites upstream and downstream of the fish farm showed no consistent increase or decrease (Figure 4.11b). The largest increase coincided with the high DON measured at site 12 in September and December 2002. One sample t-

test with a p-value of 0.98 showed no statistically significant change in DON concentration between sites 12 and 15 at Kimbridge fish farm (Table 4.2).

Table 4.2. Results from one sample t-test. Increase or decrease signifies a statistically significant change at 95 % confidence level in nutrient concentration downstream. No change indicates no statistically significant difference between the up and downstream sites. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability). Number of sample data compared indicated in brackets. The number in each box is the p-value. High p values indicate a low probability of a statistically significant difference between the two sites, and the inverse for low p values. * = Significant increase if one date removed, p-value <0.01.

	Fish farm (Kimbridge)	**Sewage treatment works (Romsey)**	**Salt marsh (lower Test)**
	(Sites 12 and 15)	**(Sites 10 and 11)**	**(Sites 2 and 4)**
Nitrate	No change (17) 0.27	Decrease (17) <0.01	No change (16) 0.29
Ammonium	No change (14) 0.74	No change (14) 0.83	No change (18) 0.63
DON	No change (14) 0.98	No change (14) 0.06	Decrease (13) 0.01
DOC	No change (14) 0.06	No change (15)* 0.37	No change (14) 0.57
PON	ND	No change (16) 0.69	No change (16) 0.24
POC	ND	No change (17) 0.79	No change 0.56

Figure 4.11c shows the DOC concentrations on each sampling date above and immediately downstream of the fish farm at Kimbridge. The DOC concentrations were usually less than 150 µM with the highest concentrations being measured on most sampling dates at site 12. This was particularly apparent from April to June 2002. Figure 4.11d confirms that a downstream increase in DOC is apparent for the majority of sampling days as only 3 values had a marked decrease and had no error bars overlapping with zero. However, the one sample t-test shows that this relationship was not statistically significant at the 95 % confidence level.

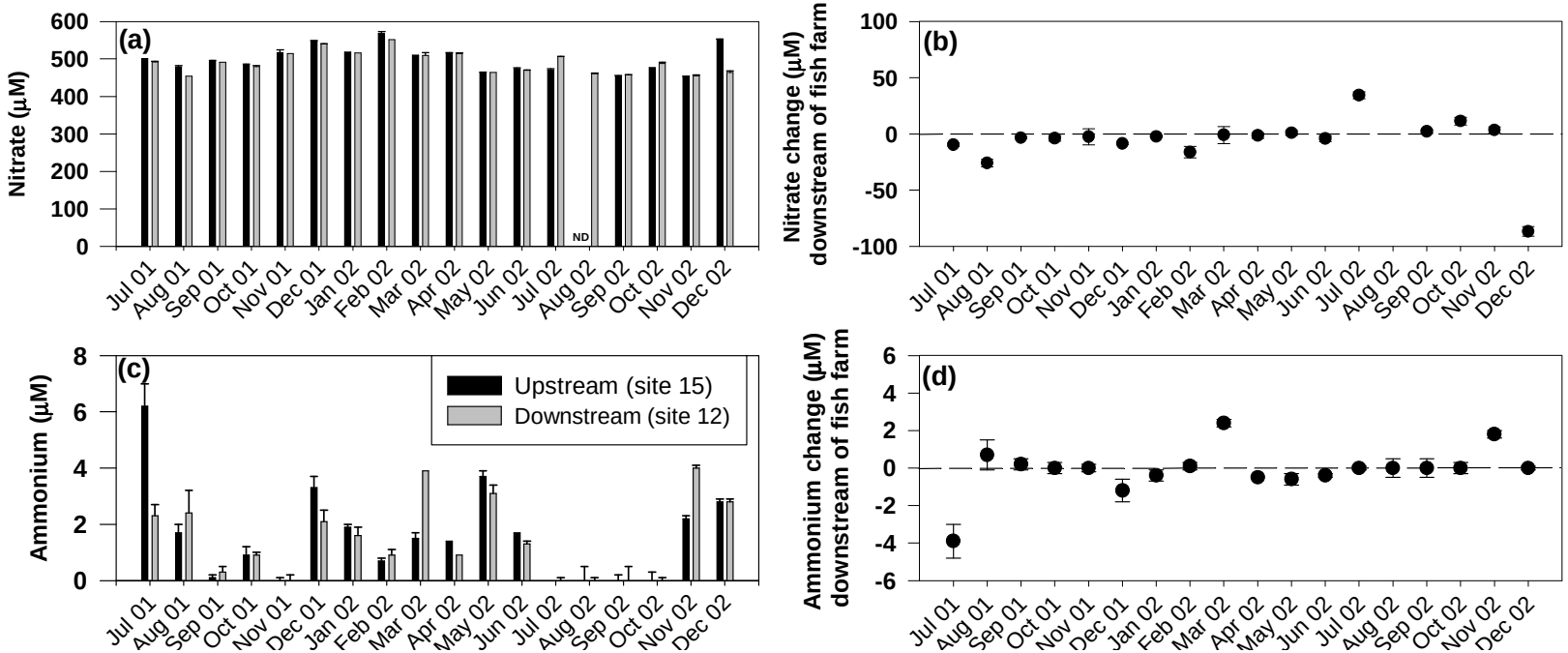


Figure 4.10 Nitrate concentrations (a) upstream (site 15) and downstream (site 12) of Kimbridge fish farm (SU 2563 3302). Error bars represent one standard deviation of 3 or 4 analytical measurements. Change in nitrate concentrations between sites 15 and 12 (b). Error bars represent compounded standard deviations from measurements taken at the two sites. Zero marked no change in concentrations downstream, positive values signified an increase downstream compared to upstream between sites, and negative values indicated a decrease. Ammonium values presented in (c) and (d) are shown in a similar way to nitrate. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

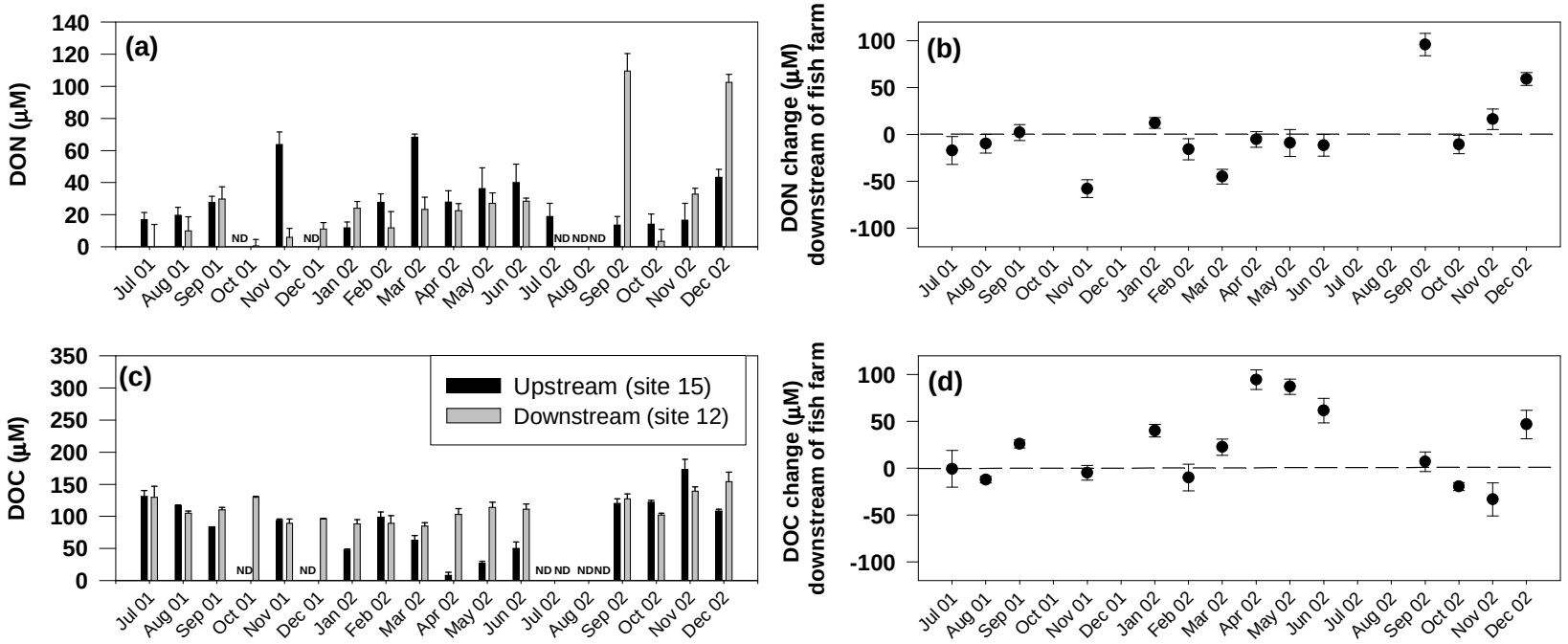


Figure 4.11 DON concentrations (a) upstream (site 15) and downstream (site 12) of Kimbridge fish farm (SU 2563 3302). Error bars represent one standard deviation of 3 or 4 analytical measurements. Change in DON concentrations between sites 15 and 12 (b). Error bars represent compounded standard deviations from measurements taken at the two sites. Zero marked no change in concentrations downstream, positive values signified an increase downstream compared to upstream between sites, and negative values indicated a decrease. DOC values presented in (c) and (d) are shown in a similar way to DON. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

Sewage treatment works at Romsey The Greenhill sewage treatment works (STW) at Romsey has a consented daily discharge of 6,410 m^3d^{-1} of secondary treated sewage (Environment Agency, 1999; Hydes, 2000). This enters the River Test via an under water pipe, immediately downstream of sample site 11. It was anticipated that this large effluent outfall would be detected in the nutrient concentrations measured downstream of the input, and influence the spatial variation in nutrients (particularly ammonium, DON and DOC). Water sampling was carried out both immediately above the outfall at the bridge at Romsey, site 11 and downstream at Longbridge, site 10 (Appendix G). Due to restricted access, this second sampling site was *ca.* 3.2 km downstream of the effluent input on the Test.

Concentrations of nitrate at site 10 showed a decrease downstream of site 11 on almost every sampling date (Figure 4.12a). This spatial variation in nitrate concentration was commented on previously in §4.3.1. and is confirmed from the change in concentrations shown in Figure 4.12b. A statistically significant decrease in nitrate concentration (p-value <0.01 at the 95 % confidence level) was measured downstream of the sewage effluent outfall (Table 4.2). This downstream change in nitrate was unlikely to be a direct result of sewage effluent input but reflects decreasing levels of nitrate along the river.

It was anticipated that ammonium concentrations would be greater downstream of the STW input in comparison with site 11 immediately above the outfall. However, no consistent change in the ammonium concentration was apparent (Figure 4.12c). The downstream change showed some temporal variation, with the summer and autumn months of both years showing a decrease in ammonium (Figure 4.12d). In contrast during winter months 2001-2002 consistent increases in ammonium concentrations were detected at site 10. There was no statistically significant change (p-value = 0.83) in the ammonium concentrations downstream from the STW (Table 4.2). Biological removal of ammonium may occur, as there was a distance of 3.2 km between the sewage effluent outfall and the sampling site.

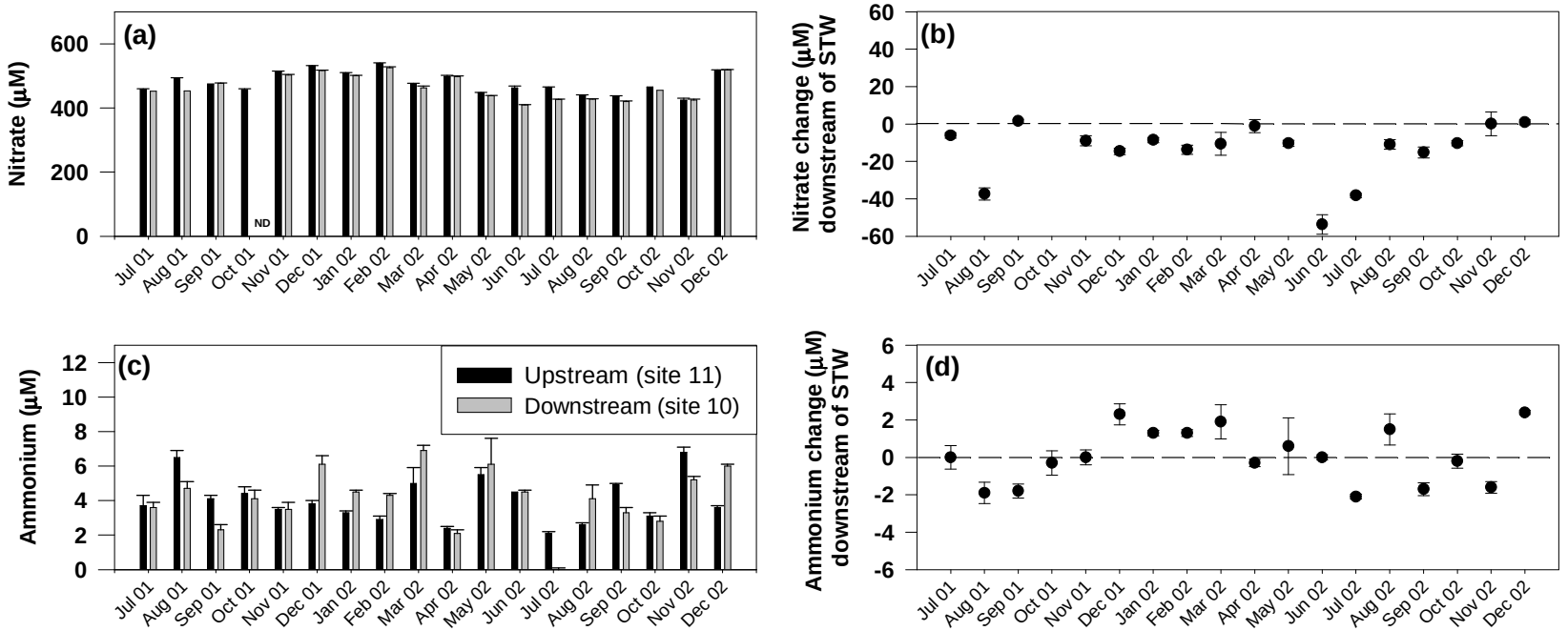


Figure 4.12 Nitrate concentrations (a) upstream (site 11) and downstream (site 10) of Romsey sewage treatment works (SU 2060 3495). Error bars represent one standard deviation of 3 or 4 analytical measurements. Change in nitrate concentrations between sites 15 and 12 (b). Error bars represent compounded standard deviations from measurements taken at the two sites. Zero marked no change in concentrations downstream, positive values signified an increase downstream compared to upstream between sites, and negative values indicated a decrease. Ammonium values presented in (c) and (d) are shown in a similar way to nitrate. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

DON concentrations above and below the input from Greenhill STW ranged from 6 to 100 µM (Figure 4.13a). An increase in concentration was measured at site 10 compared to site 11 on almost every sampling occasion. This was particularly pronounced in spring and summer 2002 (Figure 4.13b), when the increase in DON concentration downstream of the effluent input was up to 40 µM. However, the increase in DON downstream of the STW was not significant (p-value = 0.06) at the 95 % confidence level using one sample t-test (Table 4.2).

A temporal variation in DOC at sites 10 and 11 was apparent from Figure 4.13c, with higher concentrations being measured in late summer/ autumn of both years and the lowest concentrations in April and May 2002. Figure 4.13d shows that for the majority of sampling dates there was an increase in DOC concentrations between the two sites. However, concentrations of DOC were not statistically different (Table 4.2) at the 95 % confidence level, with a p-value of 0.37. The large decrease of 75 µM between sites 10 and 11 in November 2002 had a marked influence on this statistical analysis. If this data point is removed as an 'anomaly' (may be influenced by dilution effects related to the high river flow on this sampling date), the increase in DOC downstream of Greenhill STW would be statistically significant (p-value = <0.01) using a one sample t-test at the 95% confidence level.

The highest concentration of PON was measured in March and December 2002 (Figure 4.14a), and it was in these months that the largest increase in PON was recorded between sites 10 and 11 (Figure 4.14b). An increase in PON was measured on the majority of sampling dates, with the exception being June 2002 when a marked decrease in PON was observed downstream of the STW. There was no statistically significant relationship (p-value = 0.69) between the concentrations of PON above and below the effluent input from the STW (Table 4.2).

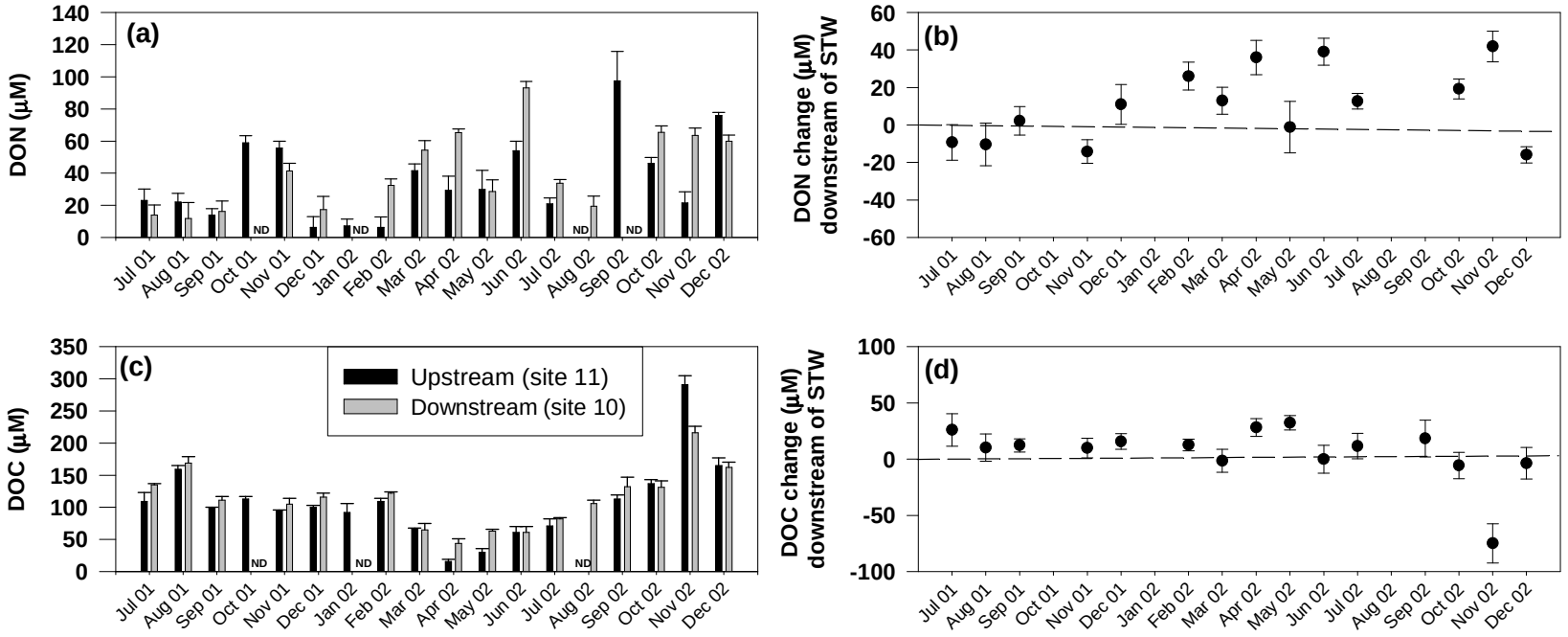


Figure 4.13 DON concentrations (a) upstream (site 11) and downstream (site 10) of Romsey sewage treatment works (SU 2060 3495). Error bars represent one standard deviation of 3 or 4 analytical measurements. Change in DON concentrations between sites 15 and 12 (b). Error bars represent compounded standard deviations from measurements taken at the two sites. Zero marked no change in concentrations downstream, positive values signified an increase downstream compared to upstream between sites, and negative values indicated a decrease. DOC values presented in (c) and (d) are shown in a similar way to DON. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

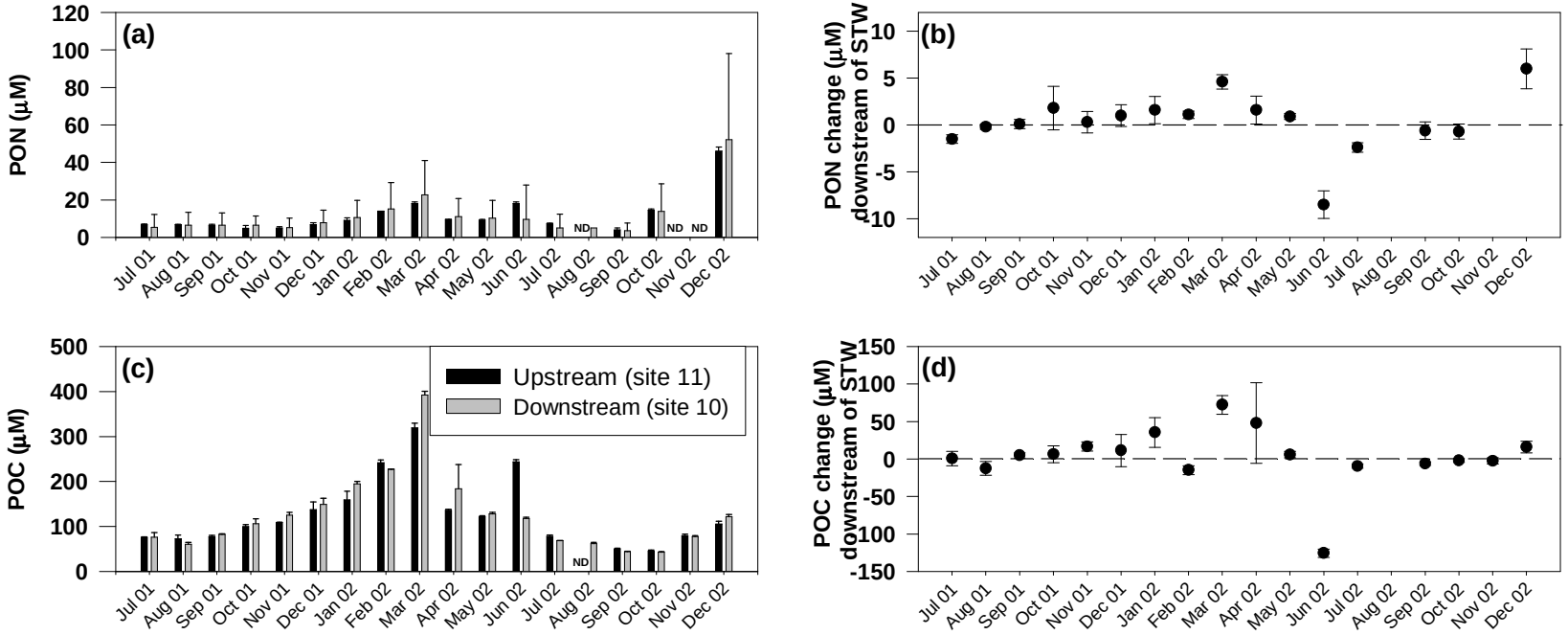


Figure 4.14 PON concentrations (a) upstream (site 11) and downstream (site 10) of Romsey sewage treatment works (SU 2060 3495). Error bars represent one standard deviation of 3 or 4 analytical measurements. Change in PON concentrations between sites 15 and 12 (b). Error bars represent compounded standard deviations from measurements taken at the two sites. Zero marked no change in concentrations downstream, positive values signified an increase downstream compared to upstream between sites, and negative values indicated a decrease. POC values presented in (c) and (d) are shown in a similar way to PON. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

The POC concentrations above and below the Greenhill STW effluent input to the River Test showed a similar pattern of temporal variation to PON (Figure 4.14c). The main difference was that POC did not show such a marked increase in December 2002 as observed for PON. In March 2002 peak POC concentrations were measured and the highest downstream increase in POC was observed. A marked decrease in POC and PON was measured in June 2002 (Figure 4.14d). On the majority of sampling dates the POC increased downstream of the STW, however when tested with a one sample t-test this relationship was not statistically significant (Table 4.2). When the large negative change recorded in June 2002 was omitted the relationship was still not statistically significant.

Lower Test salt marshes Downstream of Testwood on the lower Test (near Totton), there are extensive tidal salt marshes that stretch to Redbridge at the head of the Test estuary (Appendix H). This area has been designated as a nature reserve and access to possible sampling sites within the salt marsh was restricted, and use of a boat denied. Samples were collected on each sampling date at low tide from Redbridge (site 2) and 1.5 km upstream at Testwood (site 4). The River Test is anastomosed in the lower reaches, therefore site 4 was located on a divided channel. Although essentially a freshwater site, salinity has occasionally been measured at this site during extreme spring high tides (Testwood Fisheries manager; Pers. Comm.). With the exception of samples collected in July and August 2001, salinities measured at site 2 were <0.5, therefore the influence of salinity on any changes in nutrient concentrations was expected to be minimal. It was anticipated that changes in nutrient concentrations would be observed downstream of the salt marshes as rates of biological production and utilisation would be high in this area.

Nitrate concentrations at sites 2 and 4 had a range of 311 – 490 µM. No consistent change in concentrations between the two sites is apparent from Figure 4.15a. The change in nitrate concentrations between sites 2 and 4 is shown in Figure 4.15b and a one sample t-test showed there was no consistent, statistically significant increase or decrease (p-value = 0.29) in the nitrate concentrations below the salt marshes (Table 4.2). However temporal variation in the sign of change between the two sites, suggests that concentration changes may be important on a seasonal basis. A decrease in nitrate was measured between July and October in both years, on most sampling dates. An increase in nitrate concentrations downstream of the salt marshes was recorded between November 2001 and June 2002.

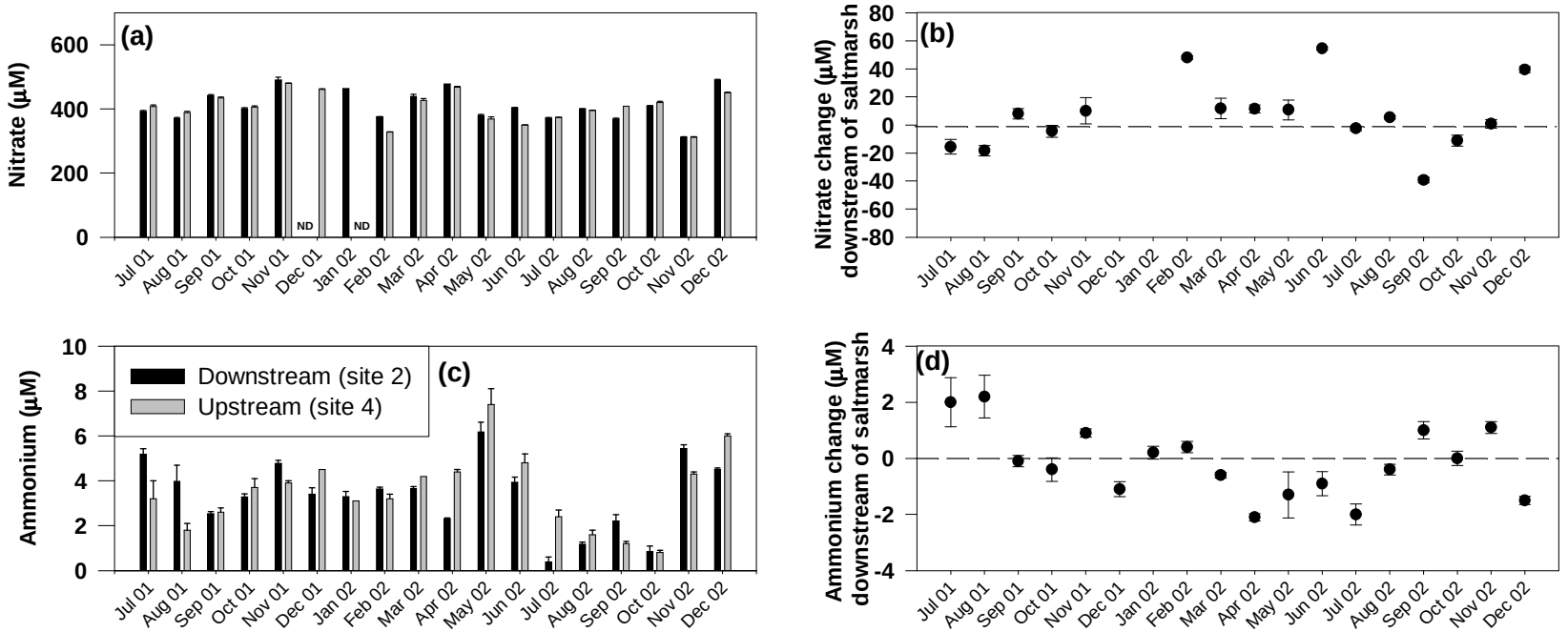


Figure 4.15 Nitrate concentrations (a) upstream (site 4) and downstream (site 2) of the salt marshes in the lower Test. Error bars represent one standard deviation of 3 or 4 analytical measurements. Change in nitrate concentrations between sites 15 and 12 (b). Error bars represent compounded standard deviations from measurements taken at the two sites. Zero marked no change in concentrations downstream, positive values signified an increase downstream compared to upstream between sites, and negative values indicated a decrease. Ammonium values presented in (c) and (d) are shown in a similar way to nitrate. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

The range of ammonium concentrations measured at sites 2 and 4 were between 0.4 and 7.4 µM (Figure 4.15c). No consistent increase or decrease in ammonium concentrations downstream of the salt marshes is apparent from Figure 4.15d. However, the changes in nitrate and ammonium concentrations showed some indication of temporal variation. The change in ammonium concentrations was opposite to that of nitrate. Exceptionally large downstream changes occurred in July and August 2001 (when salinity >0.5 was recorded); the main increases were measured downstream of the salt marshes between November 2001 and February 2002 and repeated in autumn 2002. Spring and summer 2002 had the largest decreases in ammonium concentrations downstream of site 4. This temporal signal in the concentration changes meant that no consistent and statistically significant difference was observed between the upstream and downstream sites (p-value = 0.63)(Table 4.2).

Figure 4.16a presents the DON concentrations at sites 2 and 4. A lower concentration of DON was observed on most sampling dates at site 2, downstream of the salt marshes, compared to site 4 upstream. This decrease was even recorded in February 2002 when DON concentrations in excess of 150 µM were measured at both sites. The decrease in DON at site 2, downstream of the salt marshes (Figure 4.16b), was statistically significant (p-value = 0.01). Therefore the decrease in DON concentrations between sites 4 and 2, above and below the salt marshes tested using one sample t-test could not be attributed solely to random sampling variability (Table 4.2).

The DOC concentrations at sites 2 and 4 ranged from 56 to 645 µM (Figure 4.16c). However, on the majority of sampling dates, concentrations were less than 200 µM. The change in DOC concentrations between the two sites was mostly < 100 µM (Figure 4.16d). However, in December 2001 and October 2002 a downstream increase in DOC in excess of 130 µM was observed. No statistically significant difference (p-value = 0.57) was observed between the DOC concentrations at these two sites.

Figure 4.17a shows no consistent increase or decrease in PON concentrations between sites 4 and 2. The change in concentration between these sites was temporally variable (Figure 4.17b). An increase in particulate organic nitrogen concentrations was measured downstream of site 4 in autumn 2001.

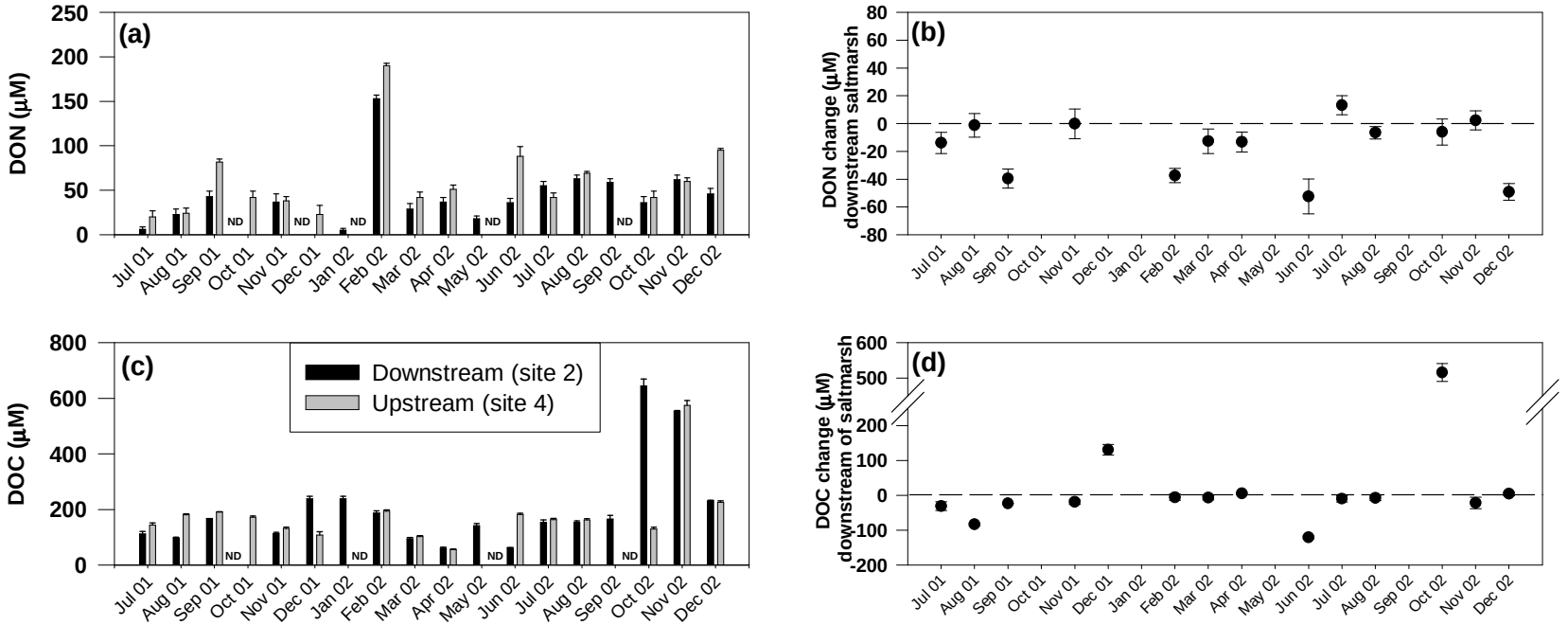


Figure 4.16 DON concentrations (a) upstream (site 4) and downstream (site 2) of the salt marshes in the lower Test. Error bars represent one standard deviation of 3 or 4 analytical measurements. Change in DON concentrations between sites 15 and 12 (b). Error bars represent compounded standard deviations from measurements taken at the two sites. Zero marked no change in concentrations downstream, positive values signified an increase downstream compared to upstream between sites, and negative values indicated a decrease. DOC values presented in (c) and (d) are shown in a similar way to DON. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

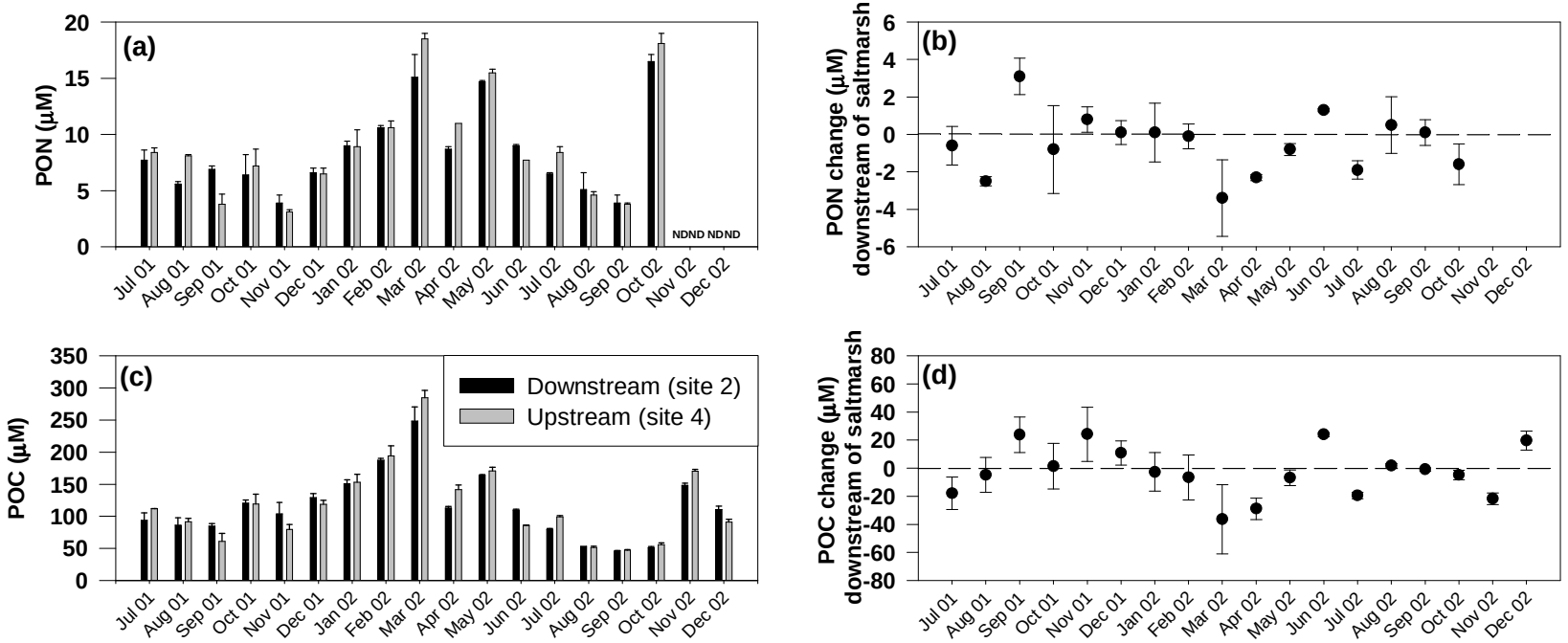


Figure 4.17 PON concentrations (a) upstream (site 4) and downstream (site 2) of the salt marshes in the lower Test. Error bars represent one standard deviation of 3 or 4 analytical measurements. Change in PON concentrations between sites 15 and 12 (b). Error bars represent compounded standard deviations from measurements taken at the two sites. Zero marked no change in concentrations downstream, positive values signified an increase downstream compared to upstream between sites, and negative values indicated a decrease. POC values presented in (c) and (d) are shown in a similar way to PON. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability).

This change between sites decreased until a maximum decrease in PON downstream of the salt marshes was observed in March 2001. The pattern continued on a roughly six monthly cycle, with an increase in PON downstream of site 4 being measured between June and August 2002. Despite this clear temporal variation in the change of PON downstream of the salt marshes, the change was not statistically significant (p-value = 0.24)(Table 4.2).

POC at sites 2 and 4 also showed a trend of change downstream as PON (Figure 4.17c and d). A downstream increase in POC between sites 4 and 2 was measured in autumn 2002 and summer 2002, but the largest downstream decrease was measured in March 2002. This temporal pattern, with decrease in POC downstream of site 4 in spring and summer, was opposite in the autumn and winter when concentrations increased downstream of site 4. Using a one sample t-test the difference between the two sites was not statistically significant (p-value = 0.56)(Table 4.2).

4.3.4 Nutrient - salinity relationship in the lower Test and upper estuary

Test estuary nutrient mixing The combination of freshwater and saline water masses in the tidal reaches of the estuary mixes water containing different concentrations of nutrients. Nutrient mixing diagrams were produced on a month-by-month basis illustrating the change in nutrient concentration with salinity at sites 18, 17, 16, 1, 2, 6 and 6a (Figure 4.18 to Figure 4.21). By presenting the data in this way it was anticipated that relationships between nutrient concentrations and salinity would become apparent. Figure 4.18 to Figure 4.21 present nutrient data from July 2001 to December 2002 for nitrate, ammonium, DON and DOC concentrations in the upper estuary / lower river region.

Linear mixing lines on the figures were estimated using the highest and lowest salinity samples in each month (where there are three or more data points). They indicate the expected change in nutrient concentration assuming linear mixing and indicate whether the nutrient mixes with salinity conservatively. A winter mixing line was estimated for nitrate and ammonium to represent a benchmark of mixing when rates of biological processes were expected to be at a minimum. These benchmarks were calculated from the mean winter freshwater and saline (salinity >30) endmember concentrations using data from November 2001 to January 2002 and November and December 2002.

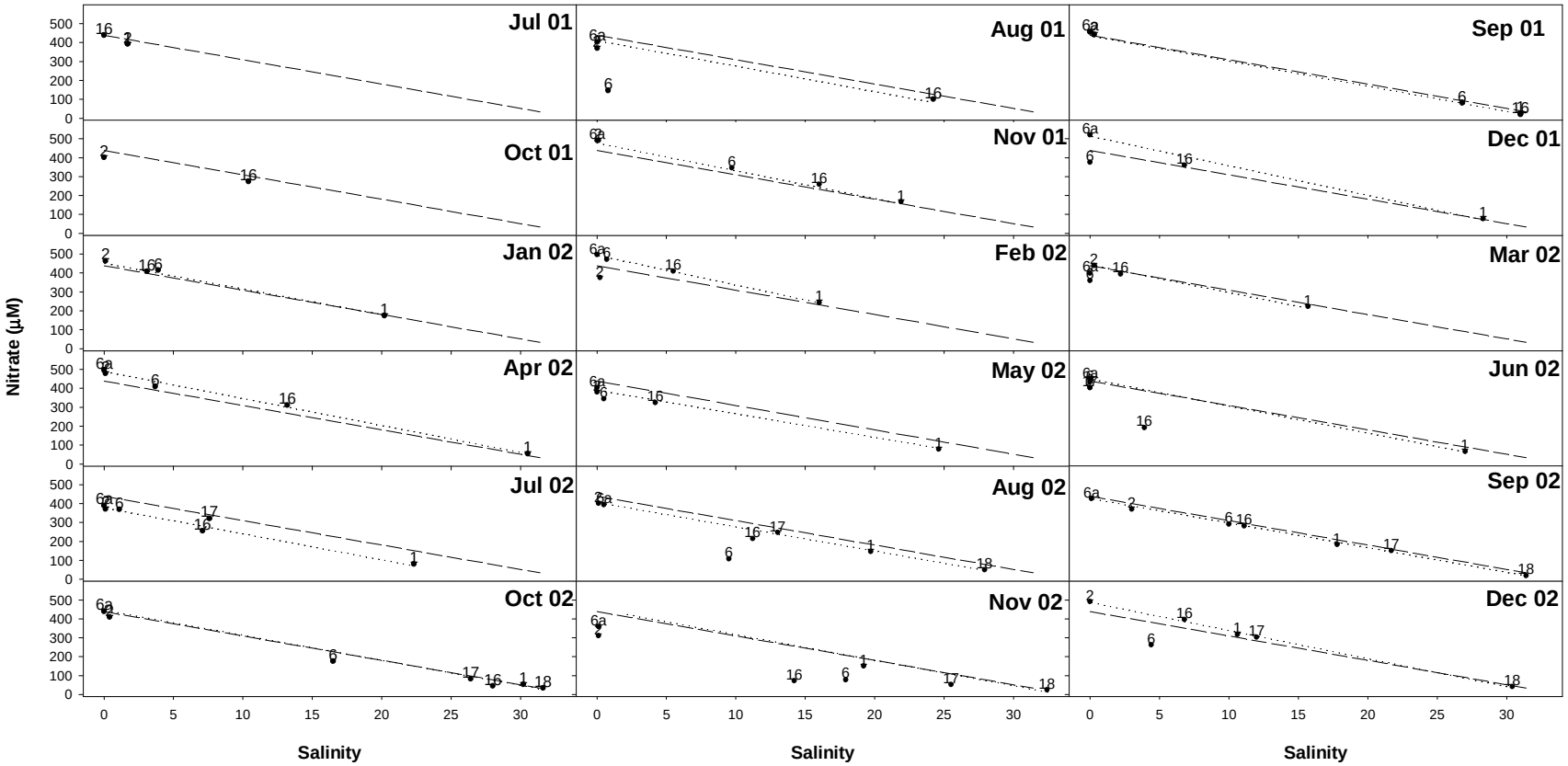


Figure 4.18 Nitrate mixing at sites 18, 17, 16, 1, 2, 6 and 6a (January 2001 - December 2002). The dashed line represents winter nutrient mixing based on winter freshwater and saline endmember concentrations. Dotted lines link the highest and lowest salinity samples (where there are 3 or more data points).

Nitrate concentrations on most sampling dates in the lower River Test and estuary varied linearly with salinity (Figure 4.18). In September 2002 the mixing at seven sampling sites was highly significant (p-value = < 0.001) when compared to the winter mixing line. The highest spring tides occurred in September 2001 and October 2002 and it was in these months that the widest range of nitrate concentrations were measured (Appendix A). The lowest neap tides were July 2001 and March 2002; the mixing diagrams for these months clearly show a narrow range of salinity in the samples. Despite the apparently linear relationship between nitrate concentration and salinity and good agreement with the winter mixing line on most sampling days, nitrate concentrations at some sample sites did not fit this pattern. Nitrate measurements did not conform to the linear mixing of the two water bodies, suggesting that removal of nitrate was occurring at these sites. For example concentrations of nitrate at site 6 were lower than the linear mixing line in August, November and December 2002. This was also observed at site 16 in June, August and November 2002. The winter mixing line lay below the dotted line of nutrient mixing between September 2001 and February 2002, whereas between May and October 2002 the winter mixing line overestimated the actual nutrient concentrations.

Concentrations of ammonium in the inter-tidal reaches of the River Test and estuary are presented in Figure 4.19. Ammonium concentrations showed a positive relationship with salinity, indicating that higher concentrations existed in the saline samples than in the freshwater samples. The clearest increases in ammonium with salinity could be observed when nutrients were sampled on the highest tides (September 2001 and October 2002). Ammonium concentrations tended to lie below the winter mixing line for most months, with the exception of July to October 2002 when the highest ammonium concentrations were measured. The most linear change in ammonium concentrations was observed in November 2001 and December 2002. The relationship between ammonium and salinity in December 2002 was statistically significant with a p-value of 0.046.

Salinity mixing diagrams for DON in Figure 4.20 show little or no relationship between DON concentration and salinity. A weak relationship was observed during the summer and autumn months, particularly in August and November 2002, and appeared to be independent of the tidal range.

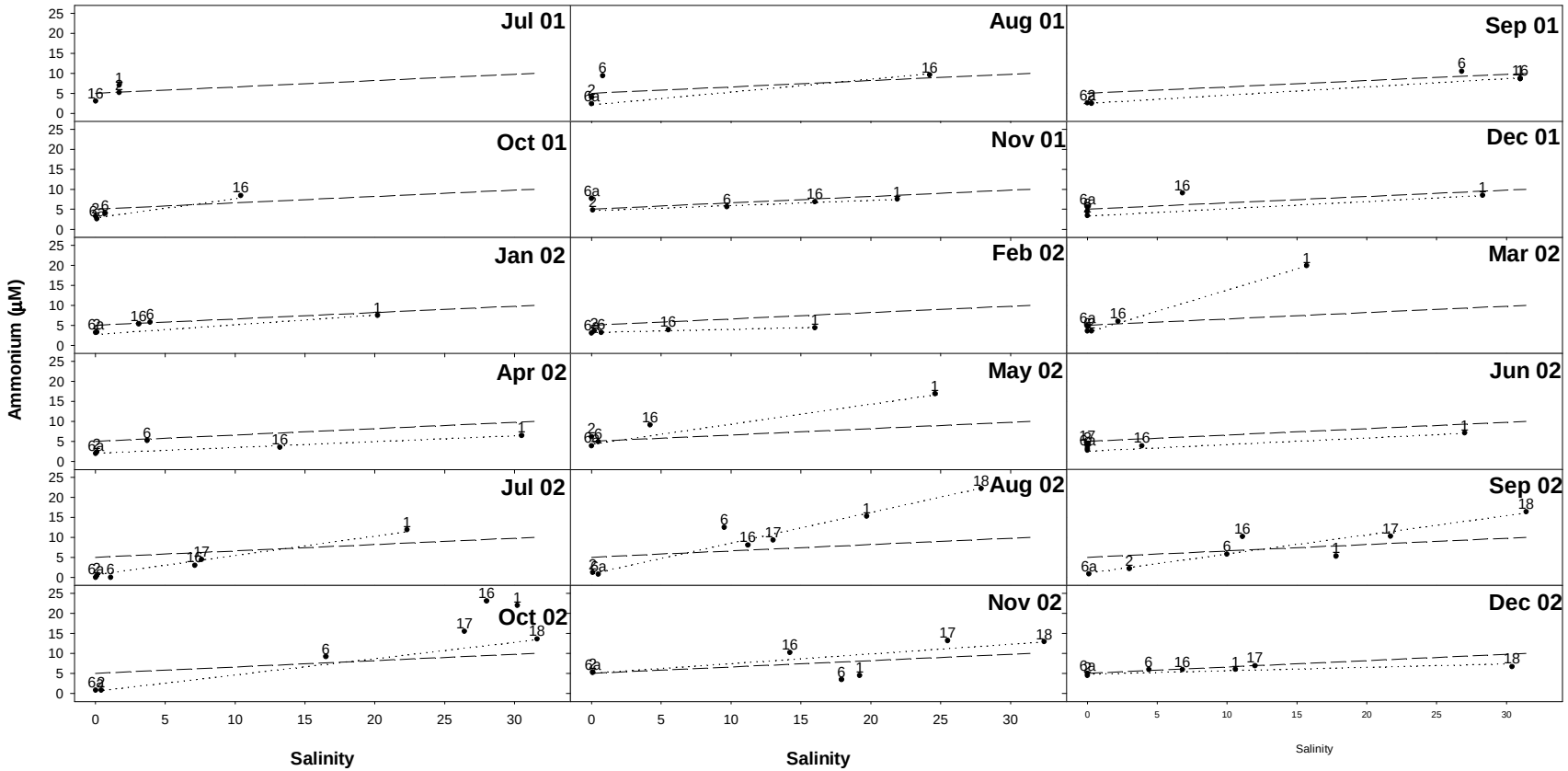


Figure 4.19 Ammonium mixing at sites 18, 17, 16, 1, 2, 6 and 6a (January 2001 - December 2002). The dashed line represents winter nutrient mixing based on winter freshwater and saline endmember concentrations. Dotted lines link the highest and lowest salinity samples (where there are 3 or more data points).

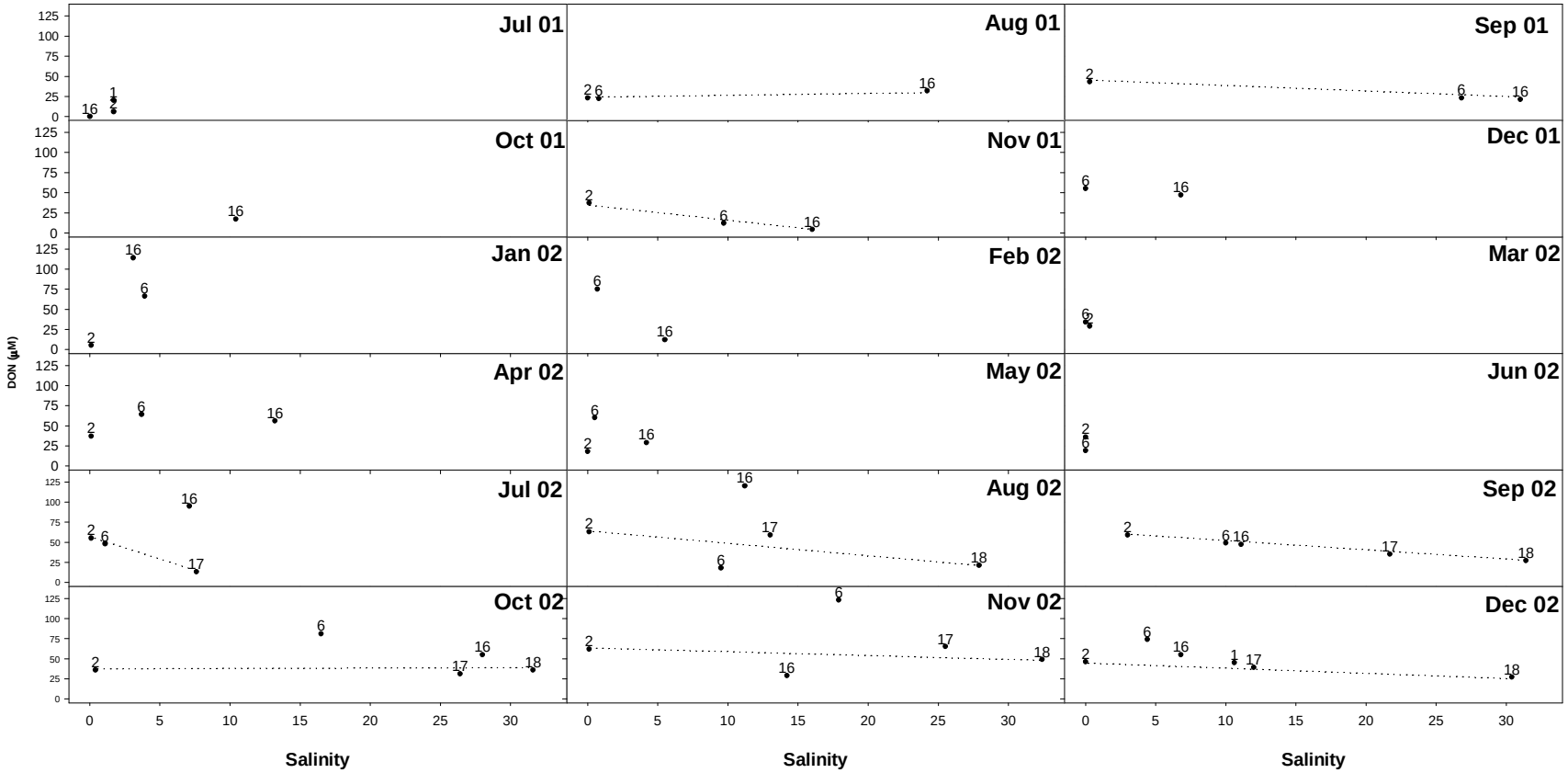


Figure 4.20 DON mixing at sites 18, 17, 16, 1, 2, 6 and 6a (January 2001 - December 2002). Dotted lines link the highest and lowest salinity samples (where there are 3 or more data points).

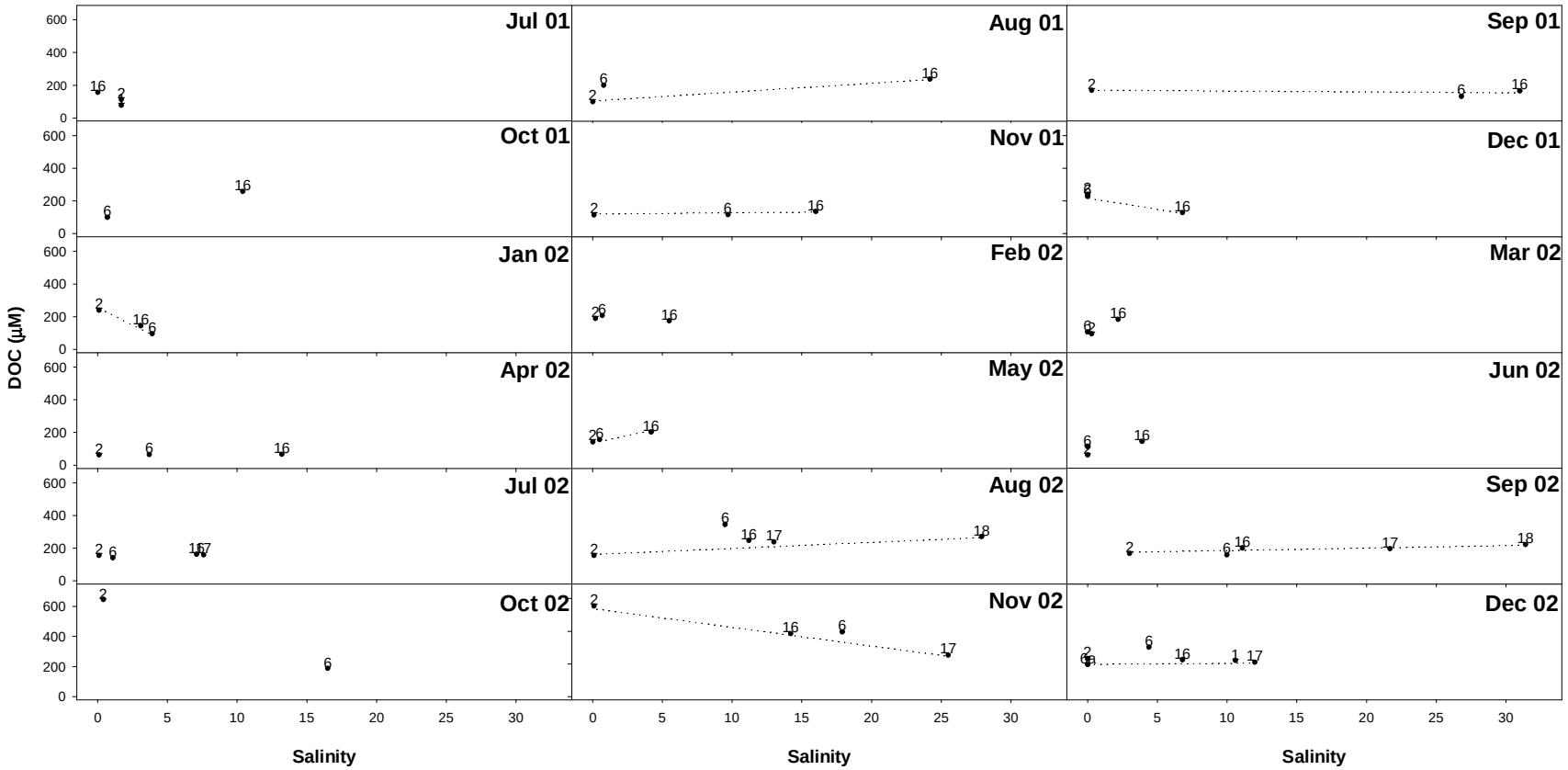


Figure 4.21 DOC mixing at sites 18, 17, 16, 1, 2, 6 and 6a (January 2001 - December 2002). Dotted lines indicate the mixing relationship between 3 or more data points.

When a relationship was observed, generally higher concentrations of DON were measured in the freshwater than in the saline samples. From October to December 2002, site 6 had consistently higher concentrations of DON than expected from the linear mixing line.

DOC concentrations plotted against salinity are presented in Figure 4.21. A weak relationship between DOC concentrations and salinity was observed on several sampling days. This indicated that higher concentrations of DOC were present in the freshwater samples and mixed with saline water containing lower concentrations of DOC. This relationship was strongest in late autumn and winter months (December 2001, January and November 2002). However, mixing between the freshwater and saltwater on other sampling dates that suggested that this trend was temporally variable. For example in August 2002 a gradual increase in DOC concentrations was measured with increasing salinity. The remaining months had fairly uniform concentrations throughout the salinity gradient, for example in September 2002.

Tidal surveys at Redbridge, Summer 2001

During the summer of 2001, two twelve-hour tidal surveys were carried out at Redbridge, both on spring tides, one with a midday low tide and the other with a midday high tide (Figure 2.1) (Appendix M). The aim of these surveys was to determine the influence of tidal variations on nutrient concentrations in the River Test / estuary interface. Samples were collected at Redbridge on a half-hourly basis for the duration of the survey and synchronous freshwater samples were collected at two hourly intervals from site 9 on the main channel (adjacent to Broadlands Lake), 4 km upstream of Redbridge (Appendix H). This continuous monitoring of freshwater inputs was conducted to identify any freshwater nutrient changes during the course of the tidal surveys.

Environmental variables 24th July 2001 The first tidal survey was carried out between 07:30 (GMT) and 18:00 on 24th July 2001, with the first high tide at 13:23. A YSI 6600 multi-probe was placed towards the base of the water column at Redbridge to give continuous readings of environmental parameters on a 30 second basis throughout the survey. A calibration was not carried out for chlorophyll a therefore nominal values (i.e. raw fluorometry data) rather than concentrations are represented in this section.

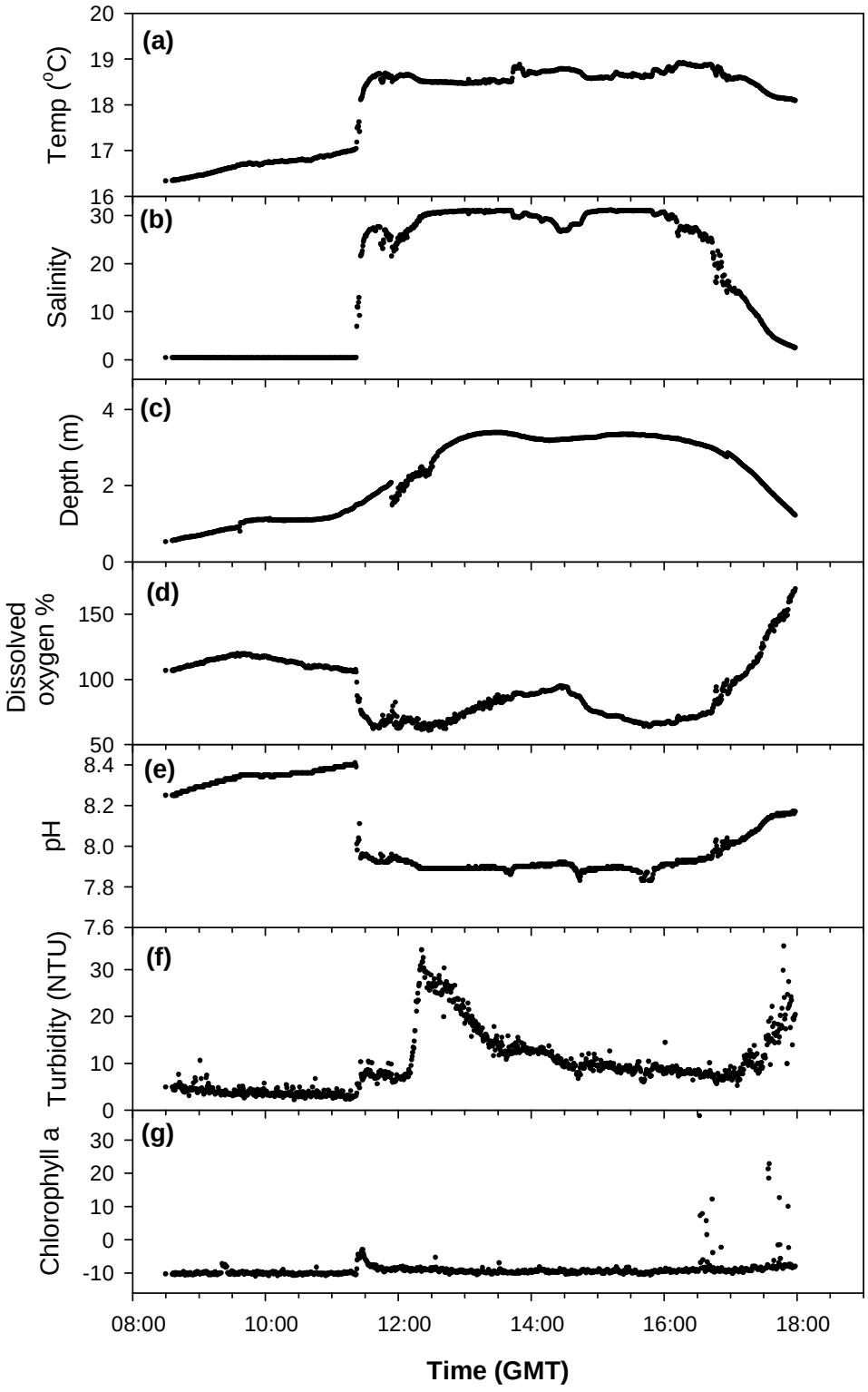


Figure 4.22. Data from YSI 6600 multi probe during a tidal cycle survey on 21st July 2001. 30-second interval data shown for (a) temperature, (b) salinity, (c) depth, (d) dissolved oxygen %, (e) pH (f) turbidity and (g) chlorophyll a. All times are given in GMT. Non zero y axes indicate nominal units.

At a stable salinity of 0 there was a very gradual increase in temperature until 11:15 when a marked increase in both parameters was recorded (Figure 4.22a and b). A prolonged period of high tide occurred between 11:30 and 17:15, (the "high water stand") and coincided with elevated water temperatures and a decline in salinity at 14:15. Towards the end of the survey a slight decline in temperature followed the marked decline in salinity at 17:00 as the tide ebbed.

The depth of water throughout the tidal survey was also recorded using the YSI 6600 multi-probe (Figure 4.22c). A gradual increase in depth was observed throughout the morning during the low tide until about 13:30. There was a slight decrease in depth at 13:45, coinciding with the temporary decrease in salinity. This was followed by the second high water at 15:30. The water depth then proceeded to decrease during the ebb until the end of the survey.

The dissolved oxygen (%) was above 100 % saturation during the low tide (Figure 4.22d). There was a marked decline in dissolved oxygen at 11:00, which was half an hour after the sharp increase in temperature and salinity. An apparent inverse relationship between the dissolved oxygen saturation (%) and salinity is observed and an increase to almost 100 % was detected at the mid point of the high water. The dissolved oxygen (%) increased to 170 % on the ebb of the high tide, which was much higher than measured during the previous low tide.

The pH throughout the tidal survey was between 7.8 and 8.4 (Figure 4.22e). A steady increase in pH up to 8.4 was observed until 11:00 when a marked decrease was recorded down to 7.8 coinciding with a marked decrease in dissolved oxygen (%). The pH remained fairly steady around 8 for the majority of the survey, with a gradual increase towards the end of the day, coinciding with a fall in salinity.

Turbidity measurements made during the tidal survey varied more than other environmental variables (Figure 4.22f). Initially following the low tide, the turbidity was about 5 NTU. There was a stepped increase at 11:30, coinciding with a marked change in many other parameters. However, the turbidity then levelled off for the following 45 minutes before sharply increasing to a maximum turbidity of up to 34 NTU at 12:20. Throughout the high water the turbidity declined and then remained consistent at about 8

NTU. Towards the end of the high water, as the salinity decreased, a second marked increase in turbidity was recorded with measurements up to 27 NTU.

Relative chlorophyll a concentrations were quite stable throughout the tidal survey (Figure 4.22g). The YSI 6600 was not calibrated for chlorophyll therefore the values are nominal units. A rapid increase in chlorophyll a was observed before 11:30, which coincided with the flood of the high tide. Elevated concentrations were measured on two other occasions at 16:45 and 17:45 during the ebb.

Nutrient measurements 24th July 2001 Prior to high tide the flow was dominated by freshwater and nitrate concentrations were in excess of 300 µM (Figure 4.23a). As the salinity increased, the concentrations of nitrate decreased sharply to a minimum concentration of 96 µM measured at 13:30. After the high tide, the nitrate concentrations increased gradually to >300 µM. However, nitrate concentrations did not return to their previous low tide concentrations when the salinity returned to zero. Samples collected from the river at the Broadlands Lake site were consistently around 500 µM and did not vary throughout the day. Therefore there was no change in the freshwater input in terms of nitrate concentration to the upper estuary.

Ammonium concentrations at the Broadlands Lake site were consistently *ca.* 5.5 µM throughout the day (Figure 4.23b). Further downstream at Redbridge, concentrations in the non-saline samples were much lower, *ca.* 1.5 µM. During the flood of the high tide, concentrations at Redbridge increased sharply and reached a peak of 10.2 µM at 13:30 (Figure 4.23b). Ammonium concentrations then decreased gradually with the ebbing tide. A clear similarity was observed between the decline in ammonium concentrations and the salinity.

A wide range of DON concentrations (0 to 84 µM) was recorded from samples collected at Broadlands Lake (site 9) during 24th July 2001 (Figure 4.23c). This suggested that the riverine input to the estuary was variable during the tidal survey. The DON concentrations on the rising tide were <60 µM, then peaked at 186 µM at 14:00. This peak in DON occurred half an hour later than the maximum concentrations of nitrate and ammonium. During the receding high tide, measurements of DON were initially >70 µM and gradually declined as the salinity decreased. The DON throughout the tidal survey fluctuated in a similar manner to the concentrations recorded at Broadlands Lake.

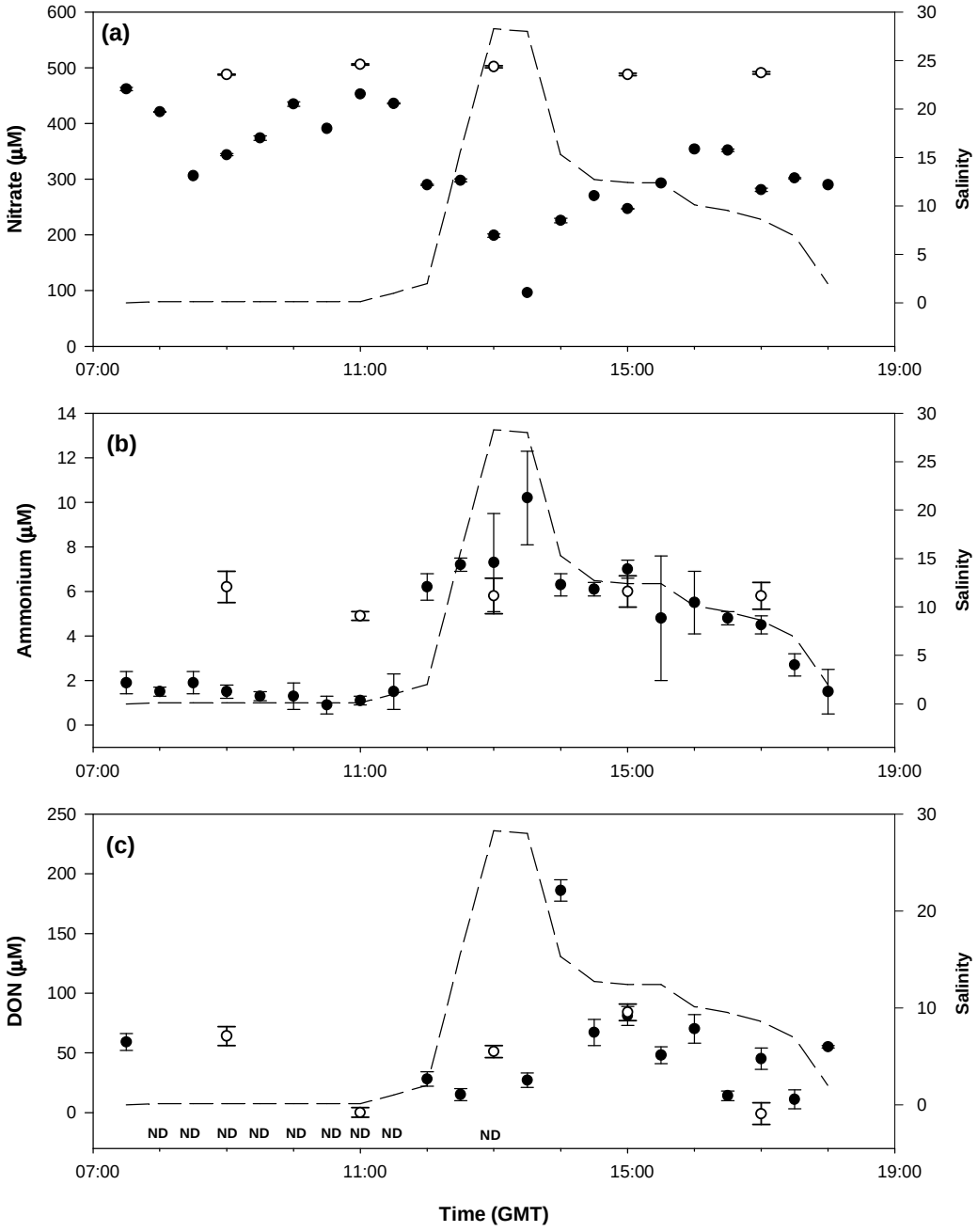


Figure 4.23. Concentrations of (a) nitrate, (b) ammonium and (c) DON during a tidal survey at Redbridge on 24th July 2001. The first low tide was at 06:58 (0.4 m), the first high water at 13:23 (4.7 m) and second low tide was at 19:21 (0.7 m). Open symbols indicate freshwater samples collected from site 9 at 2 hour intervals during the sampling day. The dashed line indicates salinity. Errors are one standard deviation of replicate analyses. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability). Underlined letters apply to samples from site 9 (Broadlands Lake).

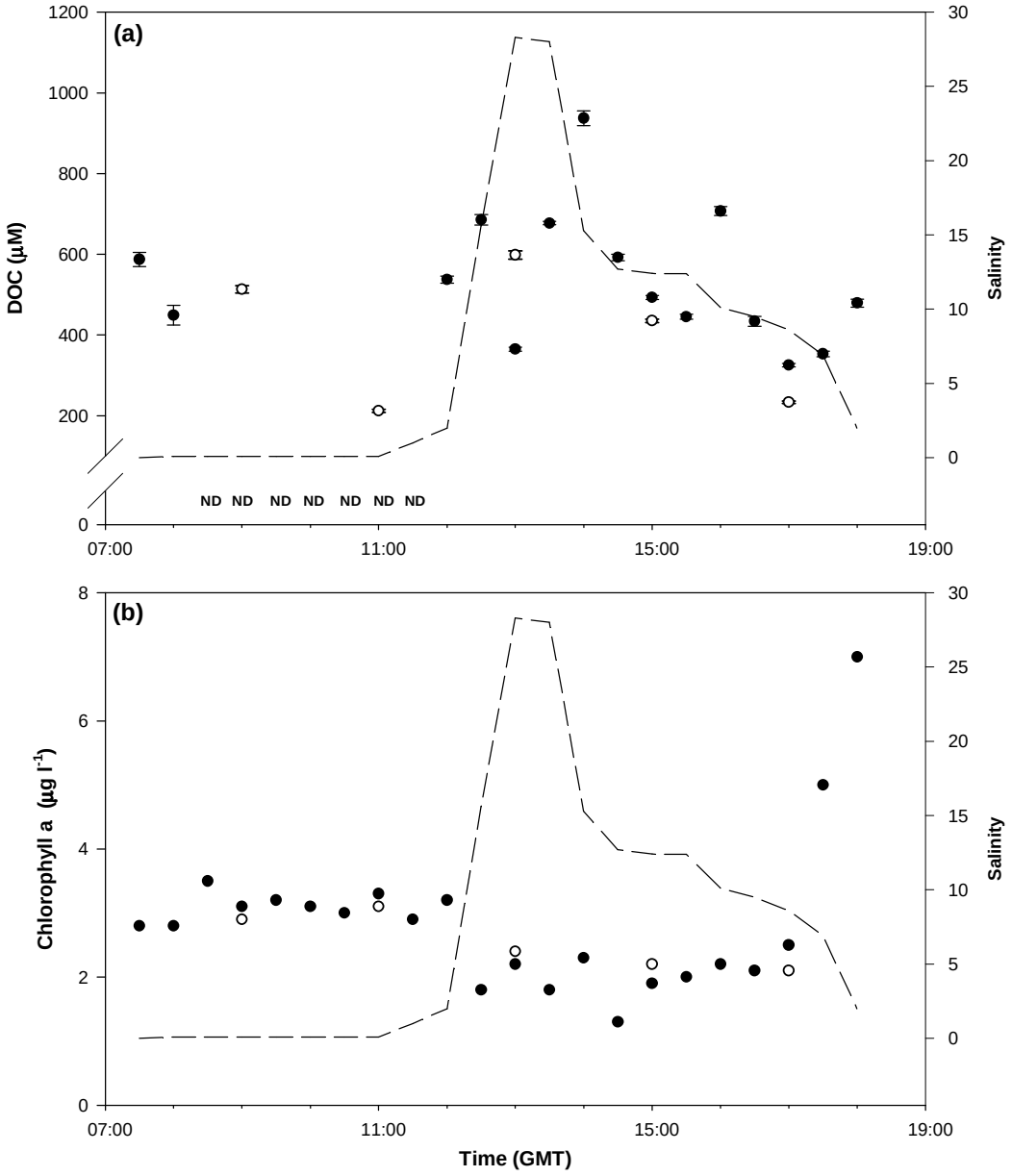


Figure 4.24. Concentrations of (a) DOC and (b) chlorophyll a during a tidal survey at Redbridge on 24th July 2001. The first low water was 06:58 (0.4 m), the first high water at 13:23 (4.7 m) and later low water was 19:21 (0.7 m). Open symbols indicate freshwater samples collected from site 9 at 2 hour intervals during the sampling day. Errors are one standard deviation of replicate analyses. The dashed line indicates salinity. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability). Underlined letters apply to samples from site 9 (Broadlands Lake).

Concentrations of DOC at Broadlands Lake during the tidal cycle were between 212 and 598 µM (Figure 4.24a). During high tide, the DOC concentration at Broadlands Lake was higher than in the saline sample. Towards the latter half of the tidal cycle the two sites had similar concentrations of DOC and concentrations were only marginally lower at Broadlands Lake. During the tidal survey, little temporal variation in the DOC concentrations was observed; the range of DOC in most samples was 325 to 707 µM. However, following the high tide stand, the DOC concentration increased to 937 µM. This peak in DOC coincided with a peak in DON at 14:00 and was half an hour later than the peak nitrate and ammonium concentrations.

Chlorophyll a concentrations during most of the tidal survey were between 1.3 and 3.5 µM (Figure 4.24b). There was close agreement between the chlorophyll a concentrations taken at Redbridge and Broadlands Lake. Following the sharp increase in salinity there was a slight decrease in the chlorophyll a concentrations, and this was observed at both Redbridge and Broadlands sites. Chlorophyll a concentrations showed a rapid increase to 5 and 7 µg l^{-1} during the ebb of the high tide, when the salinity was between 2 and 7.

Nutrient measurements 15th August 2001 A second tidal survey was carried out on 15th August 2001. The survey was again carried out from 07:30 to 18:00, but with low tide falling at mid-day. The UK Hydrographic Office tide tables predicted that the first high water would be at 06:44 (3.8 m), followed by low water at 12:44 (1.8 m) and the second water tide at 19:23 (4.0 m) (UK Hydrographic Office, 2000). The YSI 6600 multi-probe was not available for deployment during this tidal survey.

Nitrate concentrations measured during the tidal survey at Redbridge were between 356 and 468 µM (Figure 4.25a). During the initial high tide, concentrations were <400 µM but as the salinity decreased during the ebb tide, the nitrate concentrations initially fluctuated but then steadily increased after 13:00. Concentrations were generally >450 µM before the flood of the second high tide at 16:00, after which the nitrate concentrations further decreased. The riverine nitrate concentrations collected upstream from site 9 at two hourly intervals (although two samples were lost) had an average concentration of 430 ± 35 µM. A similar nitrate concentration was recorded in samples from Redbridge during low tide (salinity <1).

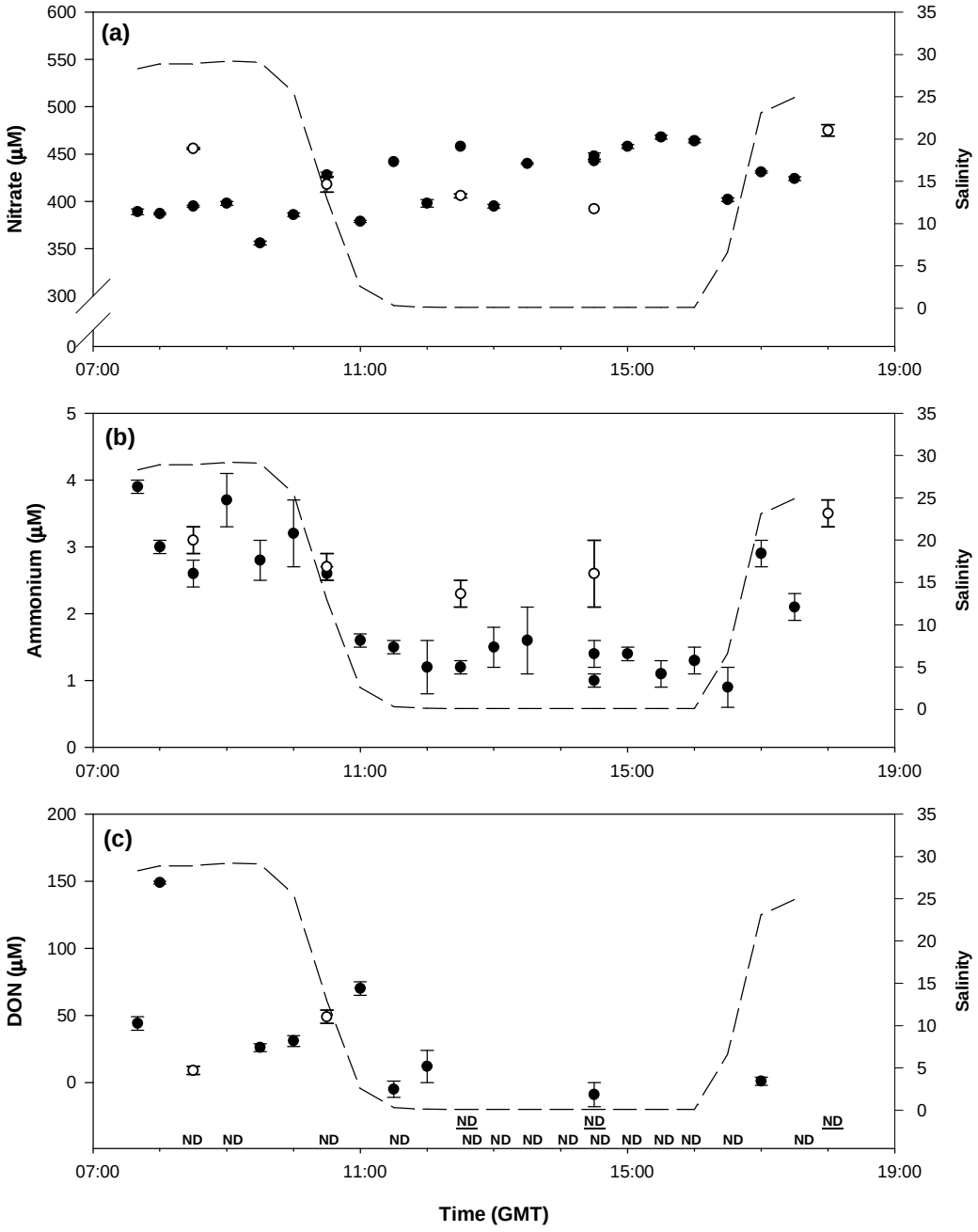


Figure 4.25. Concentrations of (a) nitrate, (b) ammonium and (c) DON during a tidal cycle at Redbridge on 15th August 2001. The first high water was at 06:44 (3.8 m), first low water at 12:44 (1.8 m) and the later high water at 19:23 (4.0 m). Dotted line indicates salinity. Open symbols indicate freshwater samples collected from site 9 at 2 hour intervals during the sampling day. Errors are one standard deviation of replicate analyses. The dashed line indicates salinity. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability). Underlined letters apply to samples from site 9 (Broadlands Lake).

The concentrations of ammonium measured at Redbridge varied with salinity during the tidal survey (Figure 4.25b). The highest concentrations (up to 4 µM) were recorded during the initial high tide and gradually declined during the ebb phase. Ammonium concentrations stabilised during low water with an average concentration of 1.3 µM. The rise in the salinity during the flood of the second high tide coincided with an increase in the ammonium concentrations. Throughout the tidal survey, ammonium at site 9 were *ca.* 3 µM, which was within the range of concentrations observed at Redbridge.

During the tidal survey, concentrations of DON were variable and showed no clear relationship with salinity (Figure 4.25c). Concentrations were generally less than 100 µM, with the exception of the sample collected at 08:00 which had a DON concentration of 159 µM. The measurements on samples collected upstream at site 9 were also variable, ranging from 9 to 49 µM.

Concentrations of DOC measured at Redbridge on 15th August 2001 were between 121 and 178 µM (Figure 4.26a). The highest concentrations of DOC were recorded in the most saline samples, concentrations falling to an average of 145 ± 7 µM prior to and during the low tide. Two lower DOC values were measured at 10:00 and 18:30 when the salinity was 29 and 25 respectively. The average concentration and standard deviation of DOC measured in the freshwater samples collected at site 9 was 130 ± 9 µM, which was lower than most samples collected at Redbridge during the tidal survey.

Chlorophyll a concentrations measured at Redbridge during the tidal cycle showed some varied with salinity changes (Figure 4.26b). Initially concentrations of 1.5 µg l^{-1} were measured prior to the decrease in salinity. During the ebb, the concentrations increased to between 2.0 and 3.7 µg l^{-1}. The chlorophyll a concentrations at Redbridge did not immediately decrease when the tide turned. Chlorophyll a concentrations at site 9 were consistent (1.7 ± 0.1 µg l^{-1}) throughout the tidal cycle survey.

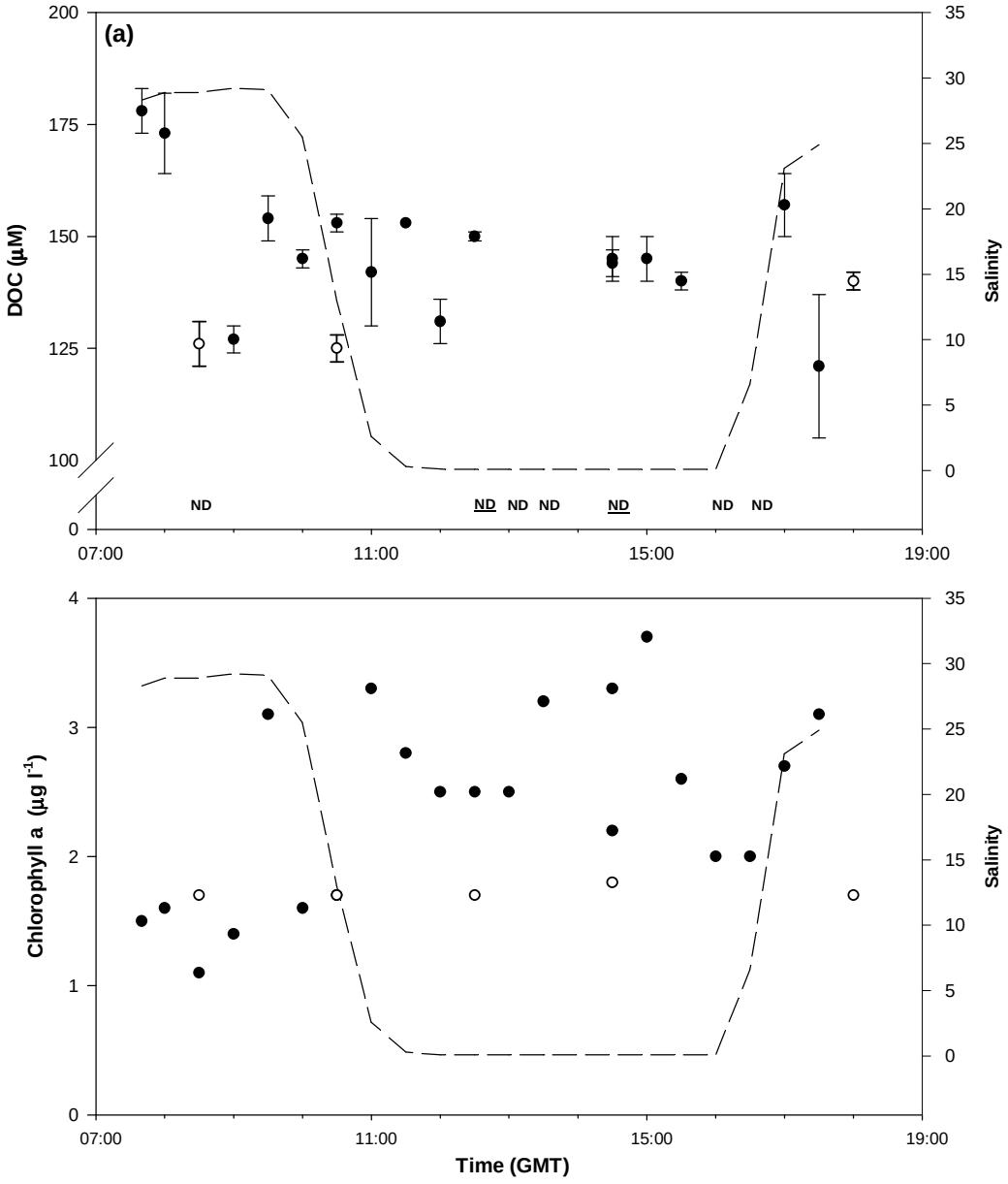


Figure 4.26. Concentrations of (a) DOC and (b) chlorophyll a during a tidal cycle at Redbridge on 15th August 2001. The first high water was at 06:44 (3.8 m), first low water at 12:44 (1.8 m) and the later high water at 19:23 (4.0 m). Dotted line indicates salinity. Open symbols indicate freshwater samples collected from site 9 at 2 hour intervals during the sampling day. The dashed line indicates salinity. ND = no data (loss of sample through bottle breakage or failure of analytical procedure to produce results of acceptable reliability). Underlined letters apply to samples from site 9 (Broadlands Lake). Error are one standard deviation of replicate analyses.

Nutrient salinity relationships during the tidal surveys In §4.3.4 nutrient-salinity plots were presented for the 18 month sampling survey. The nutrient concentrations on each sampling day were determined at a variety of sampling sites in the lower Test river and upper estuary and plotted against salinity. A more rigorous approach to establish whether linear mixing of nutrients is occurring between freshwater and estuarine water is to use data from an entire tidal cycle, collected from a single sampling point. The tidal cycle surveys at Redbridge during summer 2001 on two dates with opposing tidal cycles provided the data for this analysis. Figure 4.27 to Figure 4.28 present the tidal data in nutrient-salinity plots with dotted lines to represent the calculated mixing line. This benchmark line is derived from the extrapolation of the conservative mixing line, obtained by linear regression analysis and can be used to interpolate the calculated freshwater and saltwater endmembers.

Figure 4.27a presents the data collected on 24th July 2001 with each nutrient plotted against salinity. Nitrate concentrations during this tidal survey (with a mid-day high tide), showed some degree of nutrient mixing. The p-value of 0.01 suggested a significant linear relationship, although nitrate was not strictly conservative according to the calculated mixing line. The majority of samples with nitrate concentrations below the line were collected on the ebb tide. This is in agreement with the calculated freshwater end member of 388 µM, which was considerably lower than the nitrate concentrations entering the area upstream from site 9 at 495 ± 8 µM.

Ammonium concentrations measured during the July tidal survey also suggested that mixing was occurring between the freshwater and estuarine water bodies (Figure 4.27b). An inverse relationship to that seen for nitrate was observed as the highest ammonium concentrations were measured in the most saline samples. A p-value of <0.01 indicated a significant linear relationship and suggested that the incoming water contained high concentrations of ammonium possibly from effluent inputs from the sewage treatment works located at Millbrook and Slowhill Copse. There was also evidence of ammonium removal in the lower Test as a large discrepancy was observed between expected ammonium concentration from the calculated freshwater end member (1.8 µM) the mean concentration of 6 µM at freshwater site 9.

DON and salinity presented in Figure 4.27c shows a non-conservative scatter. However, some indication of a gradual increase in DON with salinity was observed, with the exception of the outlier collected at the highest salinity. However, it could be that there is no relationship between salinity and DON concentrations. DON concentrations recorded throughout the sampling day were 40 ± 39 µM. This concentration range encompasses all but the highest DON measurements taken at Redbridge during the tidal cycle.

DOC concentrations during the tidal survey on 24th July 2001 showed non-conservative scatter with salinity (Figure 4.27d). Although slightly higher DOC concentrations were recorded at mid-salinities there was no consistent trend with increasing salinity. Concentrations ranged from 325 to 937 µM, the majority of which were higher than the mean DOC measured at site 9 (Broadlands) during the same day.

The nutrient concentrations from the tidal survey carried out on 15th August 2001 were also plotted against salinity to investigate whether mixing of the nutrients was conservative during the mixing of freshwater and saline water at the fresh-saltwater interface. The nitrate concentrations measured during this mid-day low tide survey were much higher and of a narrower range than those observed in July 2001 during the mid-day high tide survey (Figure 4.28a). The nitrate concentrations varied in a similar manner to that seen in the tidal survey in July in that during the ebb tide, concentrations being generally lower than the calculated mixing line and suggesting that removal of nitrate was occurring. The mean nitrate concentration measured during the tidal survey on the main river channel at Broadlands was slightly lower than estimated by the calculated mixing line, although the range of concentrations encompassed most of those recorded at Redbridge. The mean concentration measured at site 9 was some 70 µM lower than recorded on 24th July 2001.

Ammonium concentrations were much lower in the samples collected at Redbridge during the mid-day low tide, 15th August 2001 in comparison to the survey on 24th July 2001 (Figure 4.28b). This may have been due to the July 2001 sampling being carried out during higher tides than August 2001. A gradual decrease in ammonium concentrations was observed with salinity during the ebb of the first high tide. This suggested a near conservative relationship between ammonium concentrations and salinity, which was confirmed by a calculated mixing line with a p-value of <0.01.

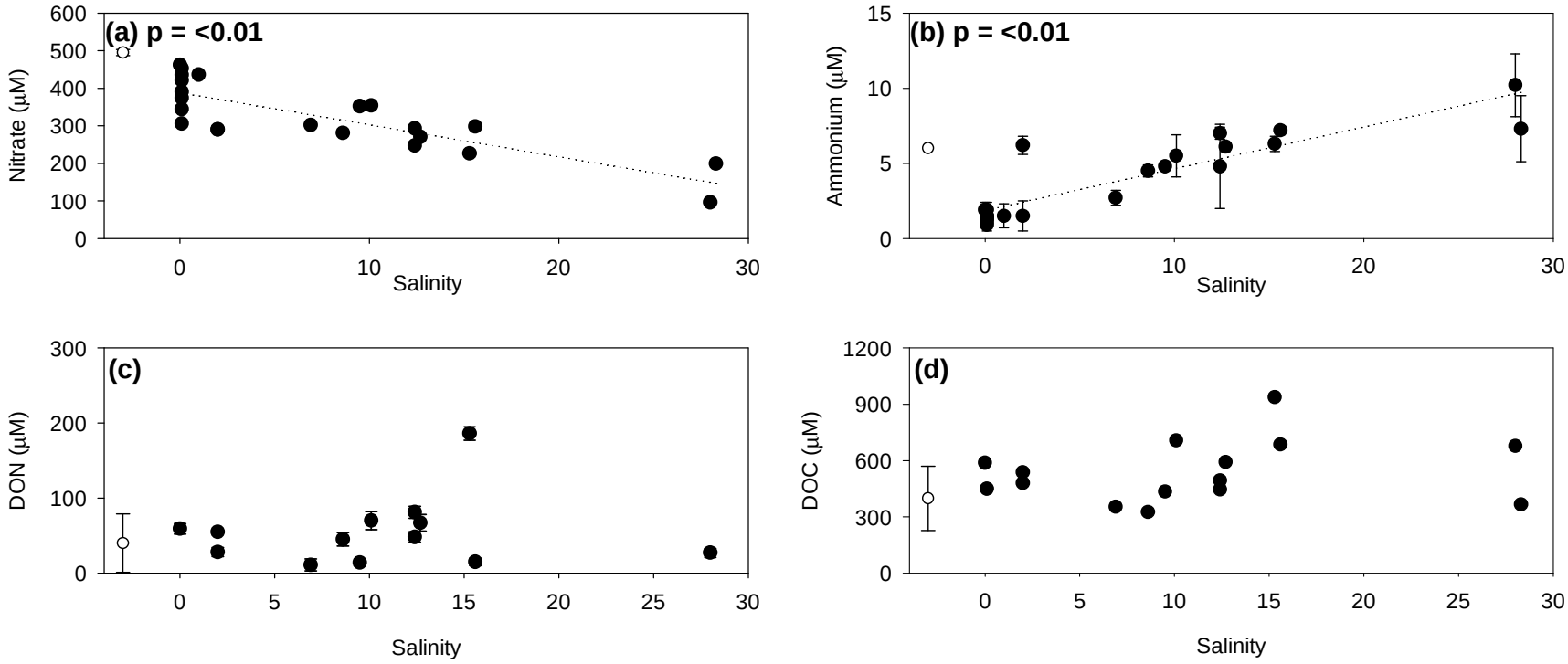


Figure 4.27 Relationship between salinity change over time at Redbridge and (a) nitrate, (b) ammonium, (c) DON and (d) DOC concentrations during the tidal cycle on 24th July 2001. The regression (dotted) line represents the calculated mixing line. Open symbols represent up to 5 measurements taken at site 9 during the course of the tidal survey.

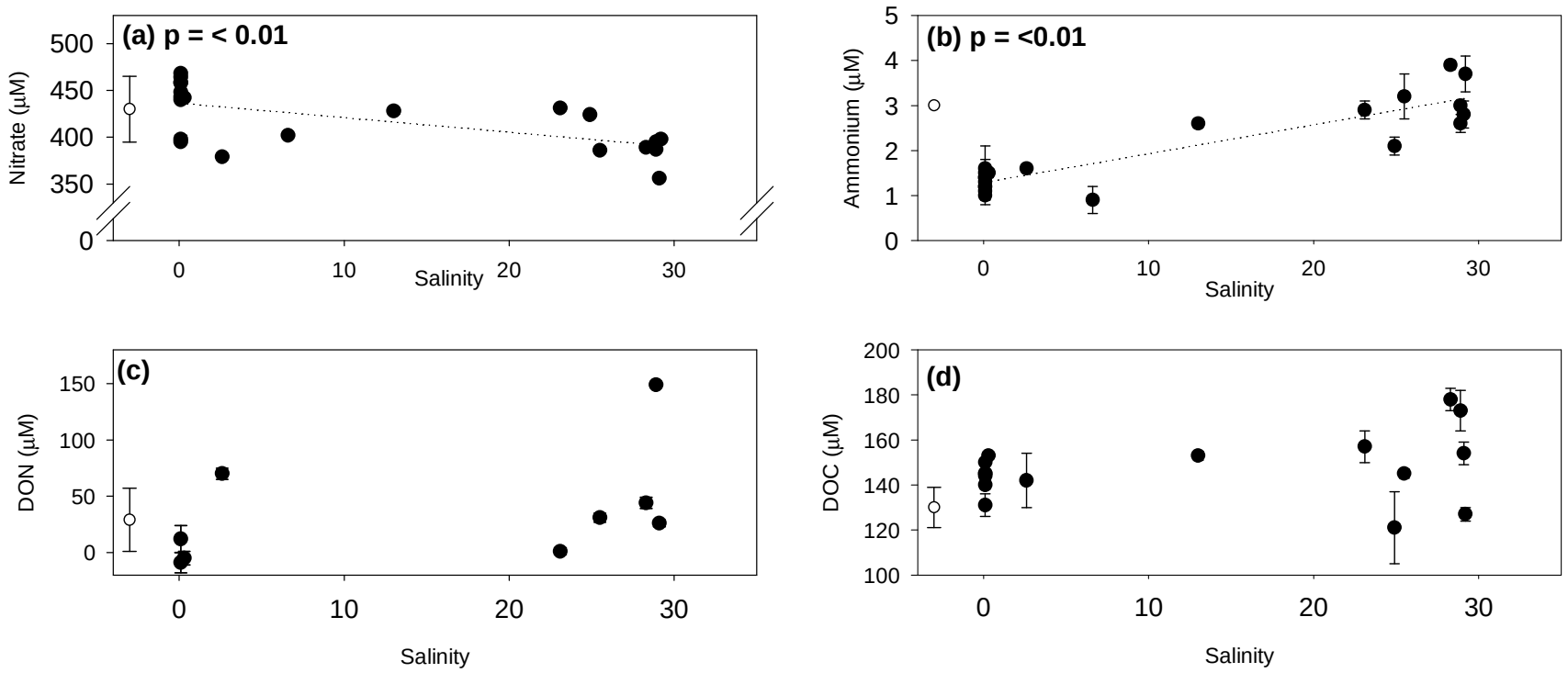


Figure 4.28 Relationship between salinity change over time at Redbridge and (a) nitrate, (b) ammonium, (c) DON and (d) DOC concentrations during the tidal cycle on 15th August 2001. The regression (dotted) line represents the calculated mixing line. Open symbols represent up to 5 measurements taken at site 9 during the course of the tidal survey.

Similar to the trend seen in July 2001, higher ammonium concentrations were measured at site 9 (3 µM) than estimated from the calculated freshwater endmember (1.2 µM). This suggested removal of ammonium between site 9 at Broadlands Lake and Redbridge. During the flood of the second high tide, the ammonium concentrations mainly fell below the calculated mixing line, suggesting that the greatest removal of ammonium occurred during this stage of the tidal cycle.

A limited number of DON measurements were available to plot against salinity from the August 2001 tidal survey (Figure 4.28c). Despite this, a similar pattern was observed as for 24th July 2001 as there appeared to be a non-conservative relationship between DON and salinity. The concentrations in the more saline samples were higher than in the freshwater samples, although few mid-salinity values for DON were available. The mean concentration of DON at site 9 was 29 ± 28 µM, similar to DON concentrations measured at both low and high salinities.

DOC concentrations measured at Redbridge during the tidal cycle showed little variation with salinity (Figure 4.28d). Concentrations were fairly stable at around 150 µM throughout the day and there was little deviation from this even at higher salinities. Mean DOC concentrations recorded at site 9 were towards the lower end of the range of values measured at Redbridge during the tidal cycle.

Summary of nutrient salinity mixing during 18 month survey The relationship between nutrient concentrations at sites 18, 17, 16, 1, 2, 6 and 6a and salinity was investigated over the 18 month sampling period. The salinity is presented in Figure 4.29 and Figure 4.30 against nitrate, ammonium, DON and DOC.

Nitrate concentrations had a quasi conservative scatter with salinity with most data values lying either on or below the calculated mixing line (Figure 4.29a). The relationship between nitrate concentration and salinity was highly significant with a p-value < 0.01. The concentrations were highest and of widest range in freshwater samples. Nitrate concentrations measured in August of both years as well as November 2002 show the largest deviation from the calculated mixing line.

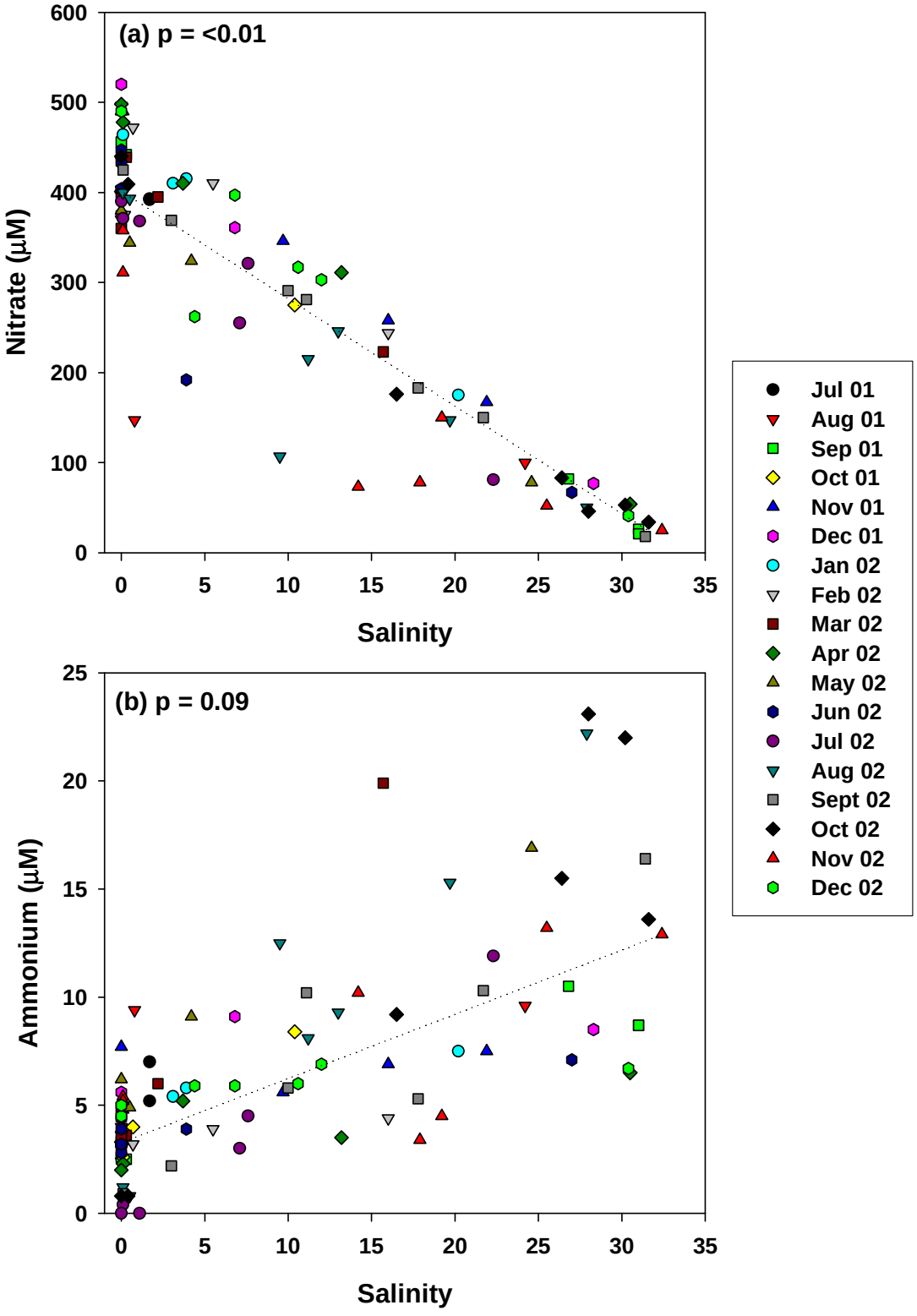


Figure 4.29. Relationship between salinity and nitrate (a) and ammonium (b) from Jul y 2001 to December 2001 at sites 18, 17, 16, 1, 2, 6 and 6a. The regression (dashed) line represents the calculated mixing line.

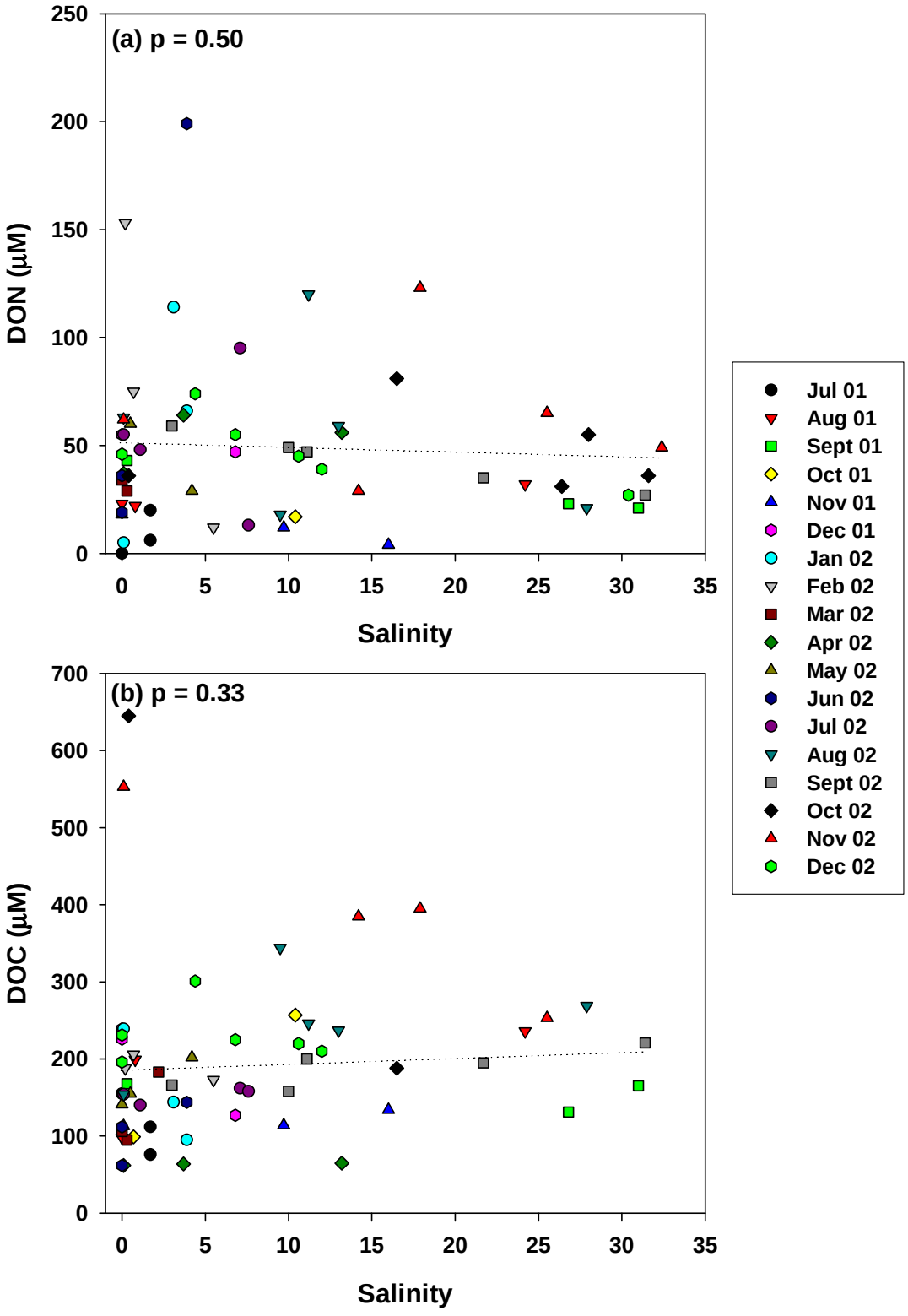


Figure 4.30. Relationship between salinity and DON (a) and DOC (b) from July 2001 to December 2001 at sites 18, 17, 16, 1, 2, 6 and 6a. The regression (dashed) line represents the calculated mixing line.

There was considerably more scatter in the relationship between ammonium concentrations and salinity than for nitrate (Figure 4.29b). The highest concentrations of ammonium were observed in the most saline samples (in October 2002) and concentrations usually <5 µM were measured at the calculated freshwater endmember. The relationship between ammonium and salinity was not significant (p-value = 0.09).

There was no statistically significant relationship (p-value = 0.50) between DON concentrations and salinity (Figure 4.30a), although concentrations of DON >100 µM were only measured in samples with salinity <17. The highest concentrations of DON were recorded in June 2002 at site 16 at a salinity of 3.9. The majority of DON samples had a concentration of <100 µM.

The relationship between DOC concentrations and salinity was similar to that of DON as the majority of concentrations were below 250 µM and had no statistically significant relationship with salinity (p-value = 0.33) (Figure 4.30b). The two highest concentrations of DOC were both recorded at zero salinity at Redbridge (site 2) in October and November 2002.

4.3.5 Multiple linear regression of spatial variations

Statistical tests were applied to data collected at three sites in the study area (sites, 4, 11 and 15) to determine which parameters contributed to the ability to predict concentrations of DON. The sites were selected to represent the upper, mid and lower study area to identify spatial differentiation. Multiple linear regression analysis was undertaken with SigmaStat software using a forward selection procedure (Kleinbaum *et al.*, 1998). Pearson product moment correlation tests were used to determine the parameter most highly correlated (i.e. highest correlation coefficient) with DON (Shaw and Purdie, 2001). Each parameter was then added in turn to the multiple linear regression equation according to decreasing correlation coefficient from the Pearson product moment correlation (Table 4.3). After the addition of each parameter the result was assessed to determine the cumulative ability to predict concentrations of DON. The multiple linear regression at all three sites showed that neither on an individual basis nor combined do these parameters have a statistically significant ability to predict concentrations of DON.

Table 4.3. Ranked parameters of decreasing correlation co-efficient from multiple linear regression of data at sites 4, 11 and 15. Value in brackets indicates Pearson product moment correlation coefficient (R^2).

	Site 4	Site 11	Site 15
Highest	DOC (0.465)	Conductivity (0.424)	POC (-0.498)
	Ammonium (0.430)	Temperature (0.358)	Conductivity (-0.426)
	Flow (0.374)	PON (0.311)	Nitrate (0.398)
	POC (-0.225)	Ammonium (0.277)	DOC (-0.322)
	Conductivity (0.200)	Chlorophyll a (-0.204)	Chlorophyll a (0.308)
	Chlorophyll a (-0.170)	Nitrate (-0.194)	Flow (0.285)
	PON (-0.163)	POC (-0.133)	Temperature (-0.199)
	Nitrate (-0.161)	Flow (0.036)	PON (0.192)
Lowest	Temperature (0.055)	DOC (0.017)	Ammonium (0.067)

4.4 DISCUSSION

Spatial variations in nutrient concentrations have been identified along the River Test to estuary continuum. Some downstream nutrient changes may be associated with terrestrial variations such as land use and geology as well as aquatic biological processes of nutrient production and consumption (Eyre and Pepperell, 1999). Effluent point sources to the river include those from anthropogenic activities such as sewage treatment works and industry. In the case of the River Test, fish farming may have the potential to input effluents to the river. The importance of nutrient changes, particularly DON and DOC, downstream of these inputs will be considered as well as the influence of salt marshes on nutrient concentrations in the tidal reaches.

4.4.1 Longitudinal changes in nutrients and environmental parameters

Relatively stable water temperatures are an important characteristic of chalk-bed river systems (Mainstone, 1999). The presence of cress farming in the upper Test catchment confirms the dependence of industry on the relatively warm water rising from the spring during the winter and relatively cools during the summer. The Environment Agency estimates the mean temperature of the Test to be 11 ± 6 ^{o}C (Environment Agency , 2002). This study found the mean water temperature during the 18 month survey to be 11.8 ^{o}C. A

gradual downstream increase in temperature was observed throughout the 18 month survey (data not presented). This is a characteristic of flowing water exposed to solaring heat as well as larger water masses downstream having a greater capacity to retain heat (Balbi, 2000; Arbuthnott, 2001).

An assessment of the downstream changes in nitrate concentrations in the study area in the River Test in Hampshire indicates strong spatial variation. There was a clear decrease in nitrate from high concentrations (600 µM) at the source to 400 µM at the lowest freshwater limit at Redbridge. High nitrate concentrations such as these have been associated with the highly populated and intensively farmed lowland regions (Oborne *et al.*, 1980; Hornung, 1999). The geology of the Test catchment, which is predominantly chalk, influences nitrate concentrations as the percolation of rainwater into the chalk aquifer produces springs of nitrate rich groundwater (Arbuthnott, 2001). Despite the downstream decrease in concentration, nitrate remained the dominant component of TN throughout the study area. Several processes are thought to explain this downstream decrease in nitrate concentration (a) dilution of ambient nitrate from river tributaries, groundwater and agricultural runoff (Black *et al.*, 1993; Heathwaite and Johnes, 1996; Neal, 2001) and (b) biological consumption of nitrate for example by biofilms, algae and macrophytes (Chapman, 1996; Pattinson *et al.*, 1998). The first process is likely to have the greatest influence during high flow conditions, particularly in the winter, whereas consumption is likely to increase during periods of higher biological productivity in spring and summer. The third potential nitrate removal process is denitrification, which is more dependent upon environmental conditions and requires sufficient organic carbon, a suitable pH and a depleted oxygen supply (Chapman, 1996; Pattinson *et al.*, 1998). A previous study carried out on the Test between 1998 and 2000 did not observe any significant longitudinal decrease in nitrate concentration, although the rivers source water was considered to be nitrate rich (Arbuthnott, 2001).

Ammonium accounted for about 1 % of TN in both freshwater and saline samples, which is within the range reported in the UK rivers (Russell *et al.*, 1998). No consistent spatial variations in ammonium concentrations were apparent along the river to estuary continuum. Research suggests that ammonium is the most variable nutrient in rivers and its spatial distribution remains poorly understood (Meybeck, 1993). Point inputs (e.g. sewage treatment works) are reported to be the most important source of ammonium (Nedwell *et al.*, 2002), although rapid denitrification occurs downstream of these point

source discharges (Russell *et al.*, 1998). No consistently significant input of ammonium was detected downstream of the sewage effluent outfall at Romsey (Greenhill STW). This will be discussed further in §4.4.3.

A downstream trend of increasing DON concentration was observed in the study area with the exception of site 2. There is an absence of studies carried out on the spatial variation in DON in freshwater environments, although there have been more extensive investigations of the relationship between DON and salinity in estuarine environments (e.g. Cauwet and Sidorov, 1996). Accumulation of DON was observed from the river source to the estuary. The absence of DON at the aquifer fed source of the River Test suggests that ground water and run off are potential diffuse sources, with biological production being an additional internal source. The decrease in DON between sites 4 and 2 upstream and downstream of the marsh were substantiated using a one sample t-test and shows that DON decrease occurs with in the salt marshes.

A similar longitudinal increase in DOC was observed in the River Test between July 2001 and December 2002 as observed for DON. Once again site 2 (downstream of the salt marshes) did not conform, suggesting that DON assimilation or ammonification processes were important in this area. Hedges *et al* (2000) observed downstream increases in DOC during a longitudinal study of the Bolivian tributaries of the Amazon River. Admittedly the altitudinal change from the first order streams to the Amazon was marked, but groundwater inputs were important in this same region (Spitzy and Leenheer, 1991) and the increase was most abrupt as the river flowed onto the flood plains and was joined by tributaries (Hedges *et al.*, 2000). Other studies have suggested that microbial processes release soil and plant derived DOC into run off via flushing processes (Spitzy and Leenheer, 1991; Tipping *et al.*, 1997). Interestingly DOC concentrations at the river source were higher than those measured at site 15 at the upper limit of the sampling area. This suggests a ground water source of DOC is diluted in the upper catchment and further downstream other DOC point source inputs such as sewage treatment works and fish farms become important (Tipping *et al.*, 1997).

POC gradually increased from 12 µM concentrations at the source to a median of 50 – 75 µM at sites 13 and 15. This increased further to a median of ca. 115 µM throughout the rest of the study area. The geology of the catchment is predominantly Cretaceous chalk, but from Mottisfont southwards (i.e. the most southern extent of the study area) there is a

change to younger Tertiary deposits (Bracklesham beds) mainly composed of sand, silts and clay, which are up to 400 m thick by the time the river reaches the estuary at Totton (Figure 1.5) (Environment Agency, 1999; 2002). It is likely that due to the permeability of Cretaceous chalk, there were only small amounts of reducible organic substances at the river source, which increases downstream to a maximum at site 4 (Pattinson *et al.*, 1998). A study on the Swale-Ouse system observed similar downstream changes in POC associated with a change from coarse sand and gravel in the headwaters to a higher proportion of silt at the downstream sites (Pattinson *et al.*, 1998). It is not possible to confirm whether this was the case in the Test as no data were collected on the amount of suspended particulate matter (SPM). Site 13 had slightly lower POC than site 15, which may be associated with the input from the River Dun about 100 m upstream of this sampling site. The geology of the River Dun catchment is mainly Tertiary London clays and Reading and Woolwich beds, which are less permeable than the Cretaceous chalk (Environment Agency, 1999). The decrease in POC downstream of this input suggests lower concentrations of POC were present in the River Dun compared to the River Test.

An increase in PON has also been observed downstream from 2 µM at the source to a median concentration of 15 µM in the main study area. This downstream increase in PON is probably associated with increased chlorophyll a and hence biological productivity. A small decrease in median PON was observed at site 2, which may be related to the influx of salinity and hence a wide range of processes occurring at this site. The lower velocities through the salt marshes below this site may account for increased deposition of particulate matter (Nedwell *et al.*, 1999).

Negligible concentrations of chlorophyll a were measured at the source of the River Test, although concentrations increased gradually downstream to a median of 5 µg l^{-1} at site 4. This indicates increasing plant biomass being measured downstream, which may be associated with changes in water temperature and water velocity. The larger water masses downstream are able to maintain a more stable temperature and dead zones also provide opportunities for algal accumulation (Balbi, 2000).

No downstream variation in DOC: DON ratio was apparent. The mean DOC: DON was 2.5 which was lower than the stoichoimetric molar relationship for new production defined in marine systems by Redfield as 6.6 (Redfield, 1958; Mari *et al.*, 2001). The results from the 18 month survey suggest that the River Test contains higher concentrations of DON

relative to DOC than predicted for marine systems by the Redfield ratio (Pakulski *et al.*, 2000). Although the application of the Redfield ratio to DOM has been questioned as there is no simple or linear relationship between DON and DOC (Hopkinson *et al.*, 1993; Kahler and Koeve, 2001).

In contrast to DOC: DON the ratio of POC: PON (between 13 and 14) was higher than the Redfield ratio of 6.6, suggesting that the particulate organic matter (POM) in the River Test was more carbon rich than originally proposed (Redfield, 1958). This may have been associated with higher atmospheric losses and solubility of carbon relative to nitrogen (Meybeck, 1982). The POC: PON may be higher than the Redfield ratio because a stronger influence of terrestrial over aquatic originated POM. Soil detritus has a C:N between 8 and 12 and terrestrial plants are often in excess of 50, compared with 6.6 for aquatic plants (Redfield, 1958; Meybeck, 1982). The POC: PON was slightly lower in the mid reaches of the study area than at the upper and lower limits of the study area. This suggested that water entering the river in the mid reaches may contain higher concentrations of POC compared to PON (Li *et al.*, 2005).

4.4.2 Upper study area – Fish farming

Aquaculture can be an effluent source to riverine and coastal environments as feed wastage, fish excretion and faeces can cause nutrient enrichment (Gowen and Bradbury, 1987; Wu, 1995; Lin and Yi, 2003). There is also potential for vitamins, antibiotics, pigments and therapeutants to impact water quality (Wu, 1995). The fish farm located at Kimbridge rears both brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) in flow through tanks and raceways which are particularly intensive aquaculture systems (Dosdat *et al.*, 1997; Tacon and Forster, 2003).

This study found no statistically significant change in nitrate, ammonium, DON or DOC downstream of the fish farm at Kimbridge (site 12) compared with site 15 immediately upstream of the farm. It is likely that a downstream change in nitrate was not observed between sites 15 and 12 as 800 m was an insufficient distance for dilution or biological consumption of nitrate to occur (Chapman, 1996; Pattinson *et al.*, 1998). Studies have reported increased concentrations of ammonium and DON in waters discharging from fish farms derived from feed wastage and feed metabolism (Lemarié *et al.*, 1998; Sakami *et al.*, 2003; Tacon and Forster, 2003). Although this study observed no downstream change in

DON it is likely that transformation to PON occurred and there may have been high sedimentation within the flow through tanks (Hall *et al.*, 1992; Wu, 1995; Burford and Williams, 2001; Trott *et al.*, 2004). In mariculture the released nutrients from caged fish farms often accumulate in the sediments and are released over long periods of time (Hall *et al.*, 1990; Hall *et al.*, 1992), although no measurements of PON or POC were taken to confirm whether this occurred in this study. The highest export of DON from the fish farm was during winter 2002 when the greatest river flows were recorded. The overflow volume from the flow through tanks probably increased and may therefore have increased the concentrations of DON entering the river (Tucker *et al.*, 1996).

The elevated concentrations of DOC observed downstream compared to upstream of the fish farm on most sample dates were probably associated with food wastage, fish excretion and faeces (Gowen and Bradbury, 1987; Wu, 1995; Lin and Yi, 2003). The largest increases occurred between April and June 2002 when the fish farm was full as a large influx of roe enters the farm at this time of year. There was decoupling of processes affecting DOC and DON in this area, as DON concentrations remained unchanged downstream of the fish farm.

The most important component of point source effluent input to the river by Kimbridge fish farm was DOC. The absence of measurable dissolved nitrogen inputs may depend on the small volume of effluent relative to river flow, which would dilute the effluent inputs. Physical and biological processes within the river are likely to cause settling of particulates, as well as rapid grazing and transformations of nutrients (Trott *et al.*, 2004).

4.4.3 Mid-study area – Sewage treatment works

Nitrate concentrations were lower downstream than upstream of the effluent input from the sewage treatment works at Romsey. The decrease in nitrate concentration downstream in the Test was likely to be due to a combination of processes including uptake by riverine flora, sediment denitrification (Pattinson *et al.*, 1998) and dilution, as shown in UK east coast rivers (Neal, 2001). Contributions of nitrate to the river from sewage effluent were probably only high during low flow conditions (Neal *et al.*, 2000a), although during the summer months when these conditions were most likely to prevail (and the greatest downstream decreases in nitrate were observed), there may have been either high denitrification or algal uptake or both (Hessen, 1999; Nedwell *et al.*, 2002).

Changes in ammonium concentrations above and below the secondary treated sewage input at Romsey were less consistent than for nitrate. The only period when consecutive months showed a downstream increase in ammonium was December 2001 to March 2002. During these winter months the rates of biological removal of ammonium were most likely at a minimum, therefore a downstream increase in ammonium was apparent. Other studies have shown that sewage treatment works are an important point source of ammonium to rivers (Balls, 1994; Servais *et al.*, 1999; Pereira-Filho *et al.*, 2001). The lack of an apparent increase in ammonium concentrations downstream of the sewage effluent input for every month was probably influenced by the 3.2 km distance between the up and downstream sampling locations (sites 10 and 11). This is a sufficient distance for appreciable nitrification of the ammonium to occur (Smith *et al.*, 1995; Russell *et al.*, 1998; Nedwell *et al.*, 2002).

A statistically significant increase in DOC, but not DON, was measured downstream of the sewage treatment works at Romsey. Elevated DOC concentrations downstream of STW have been observed in other studies as the effluents contain high concentrations of organic matter (Miller, 1999). However, limited research has been carried out on downstream changes in DON above and below point source sewage effluent inputs (Servais *et al.*, 1999; Eatherall *et al.*, 2000). Most pronounced increases were in the summer months during low flow conditions which was also observed on parts of the River Swale (Yorkshire, UK) and its tributaries (Eatherall *et al.*, 2000)

Concentrations of PON and POC increased downstream of the treated sewage input during most of the sampling period. This was probably associated with inputs of organic matter as well as elevated biological productivity associated with higher nutrient loads downstream of the point effluent source (Servais *et al.*, 1999). The largest downstream increases occurred in spring, coinciding with peak DOC and DON concentrations. These changes were not statistically significant, as a sharp decline in the downstream change was observed in the summer with rapid removal of both POC and PON downstream of the effluent input in June 2002.

4.4.4 Lower study area – Salt marshes

In the lower Test and upper estuary the use of nutrient salinity plots are useful to understand nutrient cycling as well as removal and addition processes (Sanders *et al.*, 1997). Areas of low salinity are noted to be areas of high biological productivity and involve rapid transformations between dissolved and particulate phases (Powell *et al.*, 1996; Rendell *et al.*, 1997). The upper Test Estuary contains an area of salt marshes, which are likely to affect the nutrient regimes and storage. Salt marshes are important for nitrogen recycling through rapid uptake and bacterial fixation, conversion and release, therefore contributing to nutrient export and production of organic matter (Valiela and Teal, 1979). Sediments in salt marshes are important as bioturbation can release nutrients, and high concentrations of dissolved output from the sediments may be released immediately following the low tide (Malcolm and Sivyer, 1997). Conversely, dissolved nutrients from the water are consumed and depleted during low tides until replenished by the next flood of tide (Malcolm and Sivyer, 1997).

Spatial variations of nitrate in freshwater samples show different behaviour in the lower Test compared to the rest of the river. Lower median nitrate concentrations were measured at sites 2 and 4 (above and below the salt marsh) relative to the general downstream increase throughout the study area. Between these two sites temporal variation was apparent from the magnitude and direction of downstream change. Downstream increases in nitrate concentrations were recorded in winter and spring months, suggesting nitrification was an important process in the nitrogen cycling in this salt marsh area. In the summer, a downstream decrease in nitrate was observed implying there was nitrate removal due to high biological production (Anonymous, 1983; Sanders *et al.*, 1997). This consumption of nitrate in the summer was apparent from the composition of TN in June 2002, which had the smallest fraction of TN as nitrate than at any other time or site during the 18 month survey.

Conservative behaviour of nitrate with salinity was observed for the majority of sampling dates, which is common in UK estuaries (Fichez *et al.*, 1992; Balls, 1994; Shaw *et al.*, 1998). This relationship has been observed in Southampton water since 1908 when the first nutrient analyses were carried out on the estuary (Phillips, 1980). There does not appear to be any addition of nitrate from sewage effluents; an extensive denitrification process is carried out at the sewage treatment works located on the estuary, prior to

effluent disposal (Phillips, 1980; Hydes and Wright, 1999). The widest range of salinities and most conservative behaviour was observed during the highest spring tides of the study period (September 2001 and October 2002). During spring tides the flushing time of the Test estuary is about 26 hours (compared to 75 hours for neap tides), thereby reducing the time available for modification of nutrient concentrations by internal biological processes (Balls, 1994; Rendell *et al.*, 1997; Wright *et al.*, 1997). The linear mixing line (derived from winter marine and freshwater end members) overestimates concentrations of nitrate in the summer months. This indicates possible removal of nitrate via denitrification during periods of high biological productivity. The reverse is true in winter months (e.g. November 2001 to January 2002) when higher concentrations of nitrate were measured than predicted from the linear mixing line. This is also reflected in the higher freshwater end member concentrations measured in winter compared with summer, and has been observed in other studies of the Test estuary (Hydes, 2000)

The cycling of ammonium in the lower Test and upper estuary is more complex than nitrate as it is probable that a high proportion of ammonium is derived from anthropogenic sources. A close relationship between ammonium and salinity was observed during the July 2001 tidal cycle with higher concentrations being measured in saline samples. Also a higher percentage of TN was composed of ammonium in saline than freshwater samples. This indicated there was a major source of ammonium to the estuary. Two large sewage treatment works located at Marchwood (Slow Hill Copse) and Millbrook, about 3 km downstream from Redbridge contribute to the 0.1×10^6 m^3 day^{-1} consented sewage discharge received by Southampton Water (Phillips, 1980; Hydes and Wright, 1999). The observation of higher ammonium concentrations in estuaries being associated with STW effluent inputs has been previously noted in the Rivers Tay and Dee (Balls, 1994). Sewage effluent is likely to be the reason for elevated ammonium concentrations at high salinities during the high spring tide in October 2002.

Ammonium varied quasi-conservatively with salinity in most months except summer and autumn 2002 when removal of ammonium was evident from the mixing plots; as some concentrations were lower than predicted by the linear mixing line (Figure 4.19). This suggested that other processes were occurring to alter chemically the water other than just mixing and diffusion (Thurman, 1997). This observation differed to a study conducted on the Great Ouse, UK where the greatest nutrient uptake was during the spring and summer and more conservative behaviour was observed in the winter months (Fichez *et al.*, 1992).

More conservative behaviour was expected in the winter when biological processes have less influence on nutrient cycling due to high freshwater flows reducing residence times and lower levels of biological activity (Rendell *et al.*, 1997; Alvarez-Salgado and Miller, 1998). This was reflected in the freshwater endmember concentrations from this study that showed lower ammonium concentrations during the summer months when biological removal was greatest, and higher concentrations in the winter. Similar observations have been made by previous studies on the Test (Hydes and Wright, 1999). The summer removal of ammonium downstream from site 9 to 1 was apparent from the 15th August 2001 tidal cycle carried out at Redbridge.

Generally higher concentrations of DON were recorded in freshwater samples relative to saline samples. This has been observed in other river to estuarine studies of DON on the Yealm UK (Bahr *et al.*, 2003) and Atchafalaya, USA (Pakulski *et al.*, 2000). The nutrient-salinity mixing behaviour was non-conservative in all but a couple of months. The surveys carried out in September of both years had the most conservative mixing of DON with the highest concentrations at low salinities. In September 2001 this may have been associated with the particularly high spring tide, which may have resulted in a rapid flushing of the estuary and less time for biological removal to take effect (Appendix A). However, this was not the case in September 2002 when the tide was not especially high. Non-conservative mixing of DON was observed on the majority of sampling dates, including during the summer 2001 tidal surveys. At lower salinities concentrations of DON were frequently higher than the linear mixing line suggesting that internal production of DON was possible due to the transformation of PON to DON in the tidal reaches of the salt marshes. There was also evidence of a significant downstream decrease in DON between sites 4 and 2, above and below the salt marshes, suggesting that the removal of riverine DON in the salt marshes was also important. Removal of DON may also be associated with physical processes such as adsorption, flocculation and degradation (Mantoura and Woodward, 1983; Libes, 1992; Mannino and Harvey, 2000; Uher *et al.*, 2001; Cauwet, 2002; Moreira-Turcq *et al.*, 2003).

Conservative mixing of DOC with salinity has been observed in many estuarine studies. In the UK the study of the Severn Estuary by Mantoura and Woodward (1983) suggested that the DOC was predominantly of terrigenous origin. It was probably leached from soil and consequently was composed of relatively stable humic matter that was resistant to biological transformation in the estuary (Mantoura and Woodward, 1983; Kempe *et al.*,

1991). Similar conservative behaviour of DOC was observed in the Lena River (Siberia) (Cauwet and Sidorov, 1996) and Winyah Bay (South Carolina, USA) (Goñi *et al.*, 2003). However, this mixing behaviour of DOC is inconsistent between estuaries, as many studies have also observed non-conservative mixing of DOC with salinity (Miller, 1996; Guo and Santschi, 1997; Bahr *et al.*, 2003). Results from this study agree with the non-conservative mixing observed in the Tamar, UK (Miller, 1996) and Yealm, UK (Bahr *et al.*, 2003), which indicated that DOC was either biologically reactive or occurring due to abiotic factors in these areas. The highest concentrations were generally observed at mid salinities although the two highest DOC measurements were taken from freshwater samples. Similar high DOC concentrations in freshwater samples was observed in the Yealm, UK (Bahr *et al.*, 2003) and Mississippi, USA (Pakulski *et al.*, 2000).

Losses and additions of DOC (representing non-conservative behaviour) in the upper Test Estuary can be associated with the development of the salt wedge (Goñi *et al.*, 2003) and the lower flushing rates compared with well-mixed estuaries (Cauwet, 2002). Certainly removal of DOC, possibly by the salt marshes, occurred on most sampling dates between sites 4 and 2 in the lower Test. Also lower concentrations of DOC were measured during the ebb of the 15th August 2001 tidal cycle than during the flood of the high tide, suggesting DOC removal. Similar removal processes as proposed for DON such as adsorption, flocculation and degradation are likely to occur (Mantoura and Woodward, 1983; Mannino and Harvey, 2000; Uher *et al.*, 2001; Moreira-Turcq *et al.*, 2003). Further investigations are required of DOM and particulates particularly in salt marsh areas where the settlement of particulates is likely to be important (Nedwell *et al.*, 1999). The decoupling of processes affecting concentrations of DOC and DON in the lower reaches of the Test and upper estuary suggests preferential breakdown of nitrogen-containing compounds within these salt marsh areas.

No consistent variation in POC and PON was observed between sites 4 and 2, above and below the salt marshes. This variation maybe due to seasonality in the magnitude and direction of downstream change, with removal of particulate organic matter occurring in the late winter and spring and addition of POM in the summer months (Nedwell and Trimmer, 1996). Similar temporal variation was observed by Childers *et al.* (1993) which occurred in the salt marshes of the North Inlet estuary, South Carolina.
Other variables in the lower Test and estuary have shown spatial variations. Chlorophyll a concentrations were highest immediately following the ebb of the high tide, which is

consistent with high biological productivity occurring upstream of Redbridge in the salt marsh areas. DOC: DON ratio was highest during the high tide, suggesting that higher concentrations of DOC relative to DON were present in the saline samples. This carbon-rich organic matter was likely to originate from algal material rather than terrigenous organic matter (Pakulski *et al.*, 2000; Gobler and Sanudo-Wilhelmy, 2003).

4.5 CHAPTER SUMMARY

This intensive study of spatial variations of inorganic and organic forms of nitrogen and carbon in the River Test – estuary have identified some marked downstream changes. These changes were predominantly related to external inputs from diffuse sources such as ground water and agricultural run off and point sources along the course of the river. In the lower Test and upper estuary internal production and removal of nutrients was of more importance, particularly in the salt marsh area.

Throughout the river system gradual downstream increases in DOC and DON were observed in freshwater samples, from the source to lower limits of the River Test. The intensive fish farming was neither a consistent source nor sink of DON or DOC. Sewage effluent inputs south of Romsey proved to be an important point source of DOC to the river, unlike ammonium that showed no appreciable change 3.2 km downstream.

The lower Test and estuary were influenced by the saltmarshes immediately upstream of Redbridge. This area acted as a sink for riverine DON; the reduction of DON concentration was particularly high during the spring and summer months. Non-conservative behaviour was observed for both DON and DOC during mixing with saline water, suggesting that the DOM was biologically available for cycling through production and consumption processes. The sewage treatment works located on the Test estuary were not identified as sources for DON and DOC to the upper estuary, even though they contributed to elevated ammonium concentrations in saline samples.

In conclusion although there are important point sources and sinks of DON and DOC through the River Test-estuary, diffuse sources of DOM are also present. Further investigation of these sources is required to understand fully the cycling of DOM in these river to estuarine systems.

5 CHAPTER FIVE. ASSESSING THE CHEMICAL CHARACTERISATION AND BIOAVAILABILITY OF RIVERINE DON AND DOC

5.1 INTRODUCTION

Studies of dissolved organic matter (DOM) have established that not all DOM is biologically available to phytoplankton and bacteria (e.g. Seitzinger *et al.*, 2002b). The timescales over which it becomes available may vary from minutes to years (Carlson, 2002; Cauwet, 2002). Bioavailability may depend on the chemical characteristics of the DOM and one approach is to investigate bioavailability using the molecular size composition of DON and DOC. Since DOM is composed of a heterogeneous mixture of compounds, bioavailability may depend on the molecular size composition (Bronk, 2002). In this study, attempts were made to characterise chemically the DOM using ultrafiltration techniques to separate the fractions according to molecular size. The ultrafiltration technique used two Millipore Prep/Scale™ - TFF filters at 1 and 30 kDa cut-offs to fractionate low (<1 kDa), high (>1 and <30 kDa) and very high (>30 kDa) molecular weight material. The ultrafiltered water was then used to assess the degree to which different molecular size fractions of DOM might be assimilated using a bacterial bioassay.

Although research into the chemical characterisation of DOM in many aquatic environments is ongoing, investigations into dissolved organic carbon (DOC) compounds are more advanced than that of DON (Perdue and Ritchie, 2003). This is mainly due to the relative analytical simplicity of measuring DOC compared to DON. Despite this, nitrogen is an important nutrient for aquatic biological production and requires further attention (Hedges *et al.*, 1997). A better understanding of the chemical composition of both DOC and DON is needed to assess their bioavailability to bacteria and micro algae in surface waters.

This chapter focuses on the calibration of the ultrafiltration cartridges followed by the use of bacterial bioassays for assessing DOC and DON bioavailability.

5.2 METHODOLOGY

Several techniques such as reverse osmosis and dialysis are available for the separation of DOM according to molecular size. Ultrafiltration was selected for this research as it is able

to process large volumes of feed solution, over a wide range of molecular sizes and at lower pressures than many other techniques (Cheryan, 1986; Buesseler, 1996). A Millipore Prep/Scale™ - TFF system was used to separate molecules under pressure according to molecular size to produce fractions of DOM (Ogura, 1974; Cheryan, 1986). The terminology "molecular size" rather than weight is used as the tertiary shape and orientation of molecules determines whether they are able to permeate the filter (Buesseler *et al.*, 1996). River water entered the ultrafiltration cartridge from the feed solution (Figure 5.1a) which was composed of a spiral wound membrane (Figure 5.1b). Smaller molecules were able to permeate the membrane and the larger molecules were retained and returned to the feed solution (Buesseler, 1996). This resulted in the removal of molecules that were smaller than the molecular weight cut-off of the membrane from the original solution. The larger molecules returned to the feed solution and gradually produced a more concentrated solution as the smaller molecules were removed. The degree of concentrating of the feed solution can be calculated as the concentration factor (CF) and indicates the volume reduction from the initial solution (Equation 5.1)(Guo and Santschi, 1996). The higher the concentration factor, the more concentrated the feed solution relative to the initial solution.

$$\text{Concentration factor \% (CF)} = \frac{V_o}{V_r} \qquad \text{(Equation 5.1)}$$

V_o = the volume of the original solution

V_r = the volume of retentate

In order to assess the retention of macromolecules by the membrane it is necessary to calculate the proportion of solute retained by the ultrafiltration membrane (Equation 5.2).

$$\text{Retention coefficient \% (R)} = 1 - \left(\frac{[p]}{[r]} \right) \text{x } 100 \qquad \text{(Equation 5.2)}$$

R = retention coefficient

$[p]$ = concentration of permeate

$[r]$ = concentration of retentate.

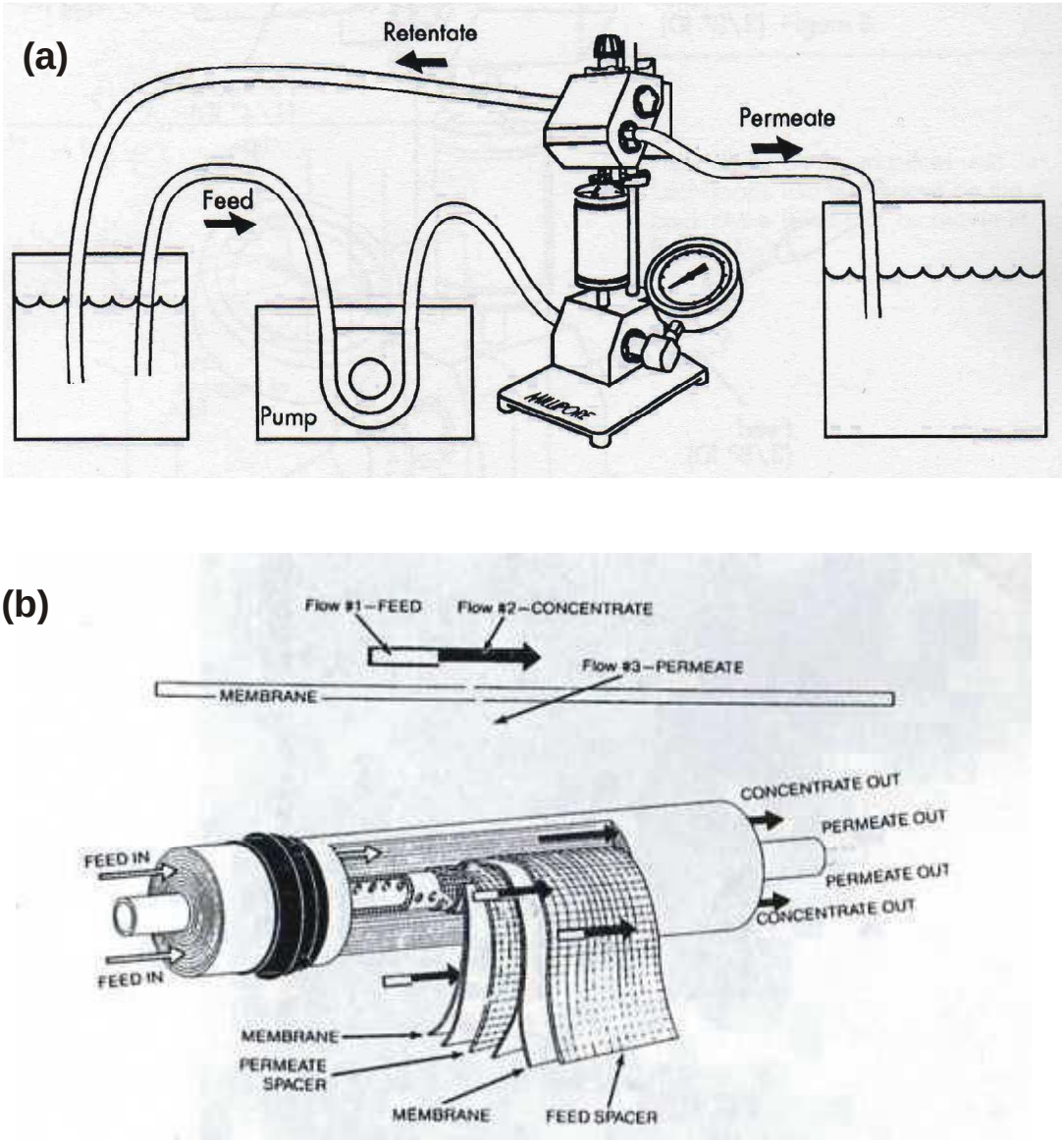


Figure 5.1. Ultrafiltration equipment used to separate DOM according to molecular size. (a) shows filter cartridge, holder and pump set up (Millipore) and (b) is a schematic of a spiral wound ultrafiltration cartridge as used in the Millipore Prep/Scale™-TFF ultrafiltration system (Cheryan, 1986) .

Filters are manufactured to a nominal molecular weight (NMW). As the actual cut-off of the filter may vary, it is important to verify the size of molecules retained by the filters using a series of macromolecules of various molecular weights (Guo and Santschi, 1996).

5.2.1 Calibration of ultrafiltration cartridges

Before fractionating river water according to molecular size, it was important to define clearly the nominal molecular weight cut-offs associated with the 1 kDa and 30 kDa ultrafiltration cartridges. A series of macromolecular substances were used as calibration standards and were selected to provide a range of molecular sizes. The macromolecules selected were raffinose (0.59 kDa), vitamin B-12 (1.33 kDa), cytochrome C (12 kDa) and albumin (66 kDa). All these macromolecules (except raffinose) contain nitrogen, therefore only DOC data are presented for calibrations involving raffinose. Two solutions of each macromolecule were prepared in milli-Q water and each pumped through either the 1 kDa or 30 kDa filter cartridge. Concentrations of DON between 40 and 60 µM were used to replicate concentrations measured in freshwater samples. The permeate and retentate were collected for later determination of dissolved organic carbon and nitrogen concentrations.

The procedure for calibrating the filters is detailed in Appendix N. Table 5.1 indicates which macromolecules were expected to permeate or be retained by the filter as an idealised outcome. Figure 5.2 to Figure 5.5 present concentrations of DOC and DON measured in the permeate and retentate at concentration factors 2, 4, 10 and 20 from both the 1 and 30 kDa filters.

Table 5.1. Idealised outcomes from the ultrafiltration of solutions of macromolecules.

	Cartridge nominal molecular size cut-off	
	1 kDa	**30 kDa**
Raffinose (0.59 kDa)	Permeate	Permeate
Vitamin B-12 (1.33 kDa)	Retentate	Permeate
Cytochrome C (12 kDa)	Retentate	Permeate
Albumin (66 kDa)	Retentate	Retentate

Albumin was the largest macromolecule used to characterise the 1 kDa filter. The DON concentrations in the permeate were less than 10 µM, which was low compared with the initial concentration of 47 µM (Figure 5.2a). A similar trend was seen in the cytochrome C, with an initial concentration of 62 µM and the concentration in the permeate not exceeding 8 µM (Figure 5.2c). This indicated that the 1 kDa filter was retaining the largest macromolecules and only a very small amount was able to pass through into the permeate. This was confirmed from measurements of DON in the retentate which indicated for both albumin (Figure 5.2b) and cytochrome C (Figure 5.2d) that a rapid concentrating of the macromolecules occurred as the concentration factor increased.

The smallest nitrogen containing molecule used to calibrate the 1 kDa filter was vitamin B-12 with a NMW of 1.33 kDa. The DON concentration in the permeate increased as the concentration factor of the retentate increased. At a concentration factor of 20 vitamin B-12 in the permeate fraction was almost equal to the initial concentration of vitamin B-12 prior to ultrafiltration (Figure 5.2e). A gradual increase in DON concentration was observed in the retentate when vitamin B-12 was passed through the 1 kDa filter, however concentrations were markedly lower than measured in the retentate for the larger macromolecules (Figure 5.2f).

Retention of the largest macromolecules was apparent from the measurements of DOC in the retentate and permeates from the 1 kDa filter. These were very similar to that observed for DON. The concentrations of DOC in the permeate from the albumin (Figure 5.3a) and cytochrome C (Figure 5.3b) standards were generally less than 23 µM. The exception was the albumin sample collected from the permeate at a concentration factor of 2. This was ten fold higher than the other permeate values, but it was only a third of the DOC concentration measured in the retentate at that time. The concentrations of DOC in the retentate showed a marked increase between the concentration factors 4 and 10 with both albumin (Figure 5.3b) and cytochrome (Figure 5.3d).

Concentrations of vitamin B-12 in the 1 kDa permeate were much higher than for albumin and cytochrome C (Figure 5.3e). As observed with the DON, the DOC concentrations in the permeate at a concentration factor of 20 were 166 µM, only 9 µM less than the initial concentration of vitamin B-12 in the initial solution. Concentrations in the retentate were much lower than measured in the retentate of albumin and cytochrome C (note the

different scale on the y-axis). However, a marked increase in DOC concentrations was observed even in the retentate at a concentration factor of 4.

The raffinose molecular size (0.59 kDa) was less than the NMW cut-off of the 1 kDa filter. It was anticipated therefore that raffinose would pass through the 1 kDa filter even at a low concentration factor. Figure 5.3g shows that even at a concentration factor of 2, the permeate concentrations were equal to those in the initial feed solution. The concentration of DOC in the retentate remained higher than in the permeate even at a concentration factor of 20 (Figure 5.3h). A stable concentration between 220 and 290 µM was obtained between the concentration factors of 10 and 20 in both the permeate and retentate fractions.

The same macromolecules were used to characterise the NMW cut-off of the 30 kDa filter. Albumin concentrations in the permeate fraction were less than 10 µM, even at the highest concentration factors (Figure 5.4a), whereas the concentration of DON in the retentate increased very gradually to a plateau between concentration factors 10 and 20 (Figure 5.4b). The albumin appears to have been retained in the retentate by the 30 kDa filter, although concentrating of the albumin in the retentate was not as apparent as in the 1 kDa retentate.

The cytochrome C was similar to albumin as the permeate concentrations were ten fold lower than the initial solution, even at a concentration factor of 20 (Figure 5.4c). However, the concentration in the retentate displayed different characteristics to any trends seen previously as the concentration of DON decreased as the concentration factor increased (Figure 5.4d). This suggests that the cytochrome C was neither crossing the filter and entering the permeate, nor being retained and concentrated in the retentate fraction. It can only be assumed that the cytochrome C molecules were retained in the 30 kDa filter cartridge.

Vitamin B-12 was the smallest nitrogen containing macromolecule used to calibrate the 30 kDa filter cartridge. Concentrations of DON in the permeate were generally greater than the initial solution (Figure 5.4e). Similar concentrations were measured in the retentate fraction (Figure 5.4f), which suggested that vitamin B-12 permeated the 30 kDa filter well.

The 30 kDa filter was characterised a second time using DOC measurements from all 4 macromolecules. Concentrations of the largest macromolecule, albumin (66 kDa) in the permeate were less than 5 % of the initial solution (Figure 5.5a). The concentration of DOC in the permeate increased slightly with increased concentration factor. The concentrations of DOC in the retentate increased more rapidly with the concentration factor (Figure 5.5b). Albumin was unable to cross the 30 kDa filter into the permeate, and it therefore became more concentrated in the retentate fraction.

The cytochrome C concentrations of DOC showed a high degree of similarity with the trends seen in the DON concentrations. The permeate concentrations remained low compared to the initial solution concentrations, even at the higher concentration factors (Figure 5.5c). In contrast the DOC concentrations in the retentate fraction decreased gradually with increasing concentration factor from the initial concentration (Figure 5.5d). The cytochrome C concentration in the retentate fraction stabilised at the higher concentration factors to two thirds of the initial concentration. This decrease in initial concentration in the retentate was observed from concentrations of both DOC and DON.

It is immediately apparent from Figure 5.5e that vitamin B-12 was not retained by the 30 kDa filter and therefore entered the permeate. The concentrations in the permeate (170 μM) even at the lowest concentration factor of 2 were greater than in the initial solution (158 μM). The concentrations of vitamin B-12 in the retentate were fairly stable even as the concentration factor increased (Figure 5.5f). The concentrating of the macromolecule as observed for albumin was not observed for vitamin B-12 in the retentate fraction.

Raffinose was the smallest macromolecule used to characterise the 30 kDa filter. The concentrations in the permeate were generally equal to the initial concentrations in the initial solution (Figure 5.5g). The exception was at concentration factor 4 when a higher concentration of raffinose was measured in the permeate than recorded in the initial solution. The concentrations of raffinose in the retentate did not change with concentration factor (Figure 5.5h).

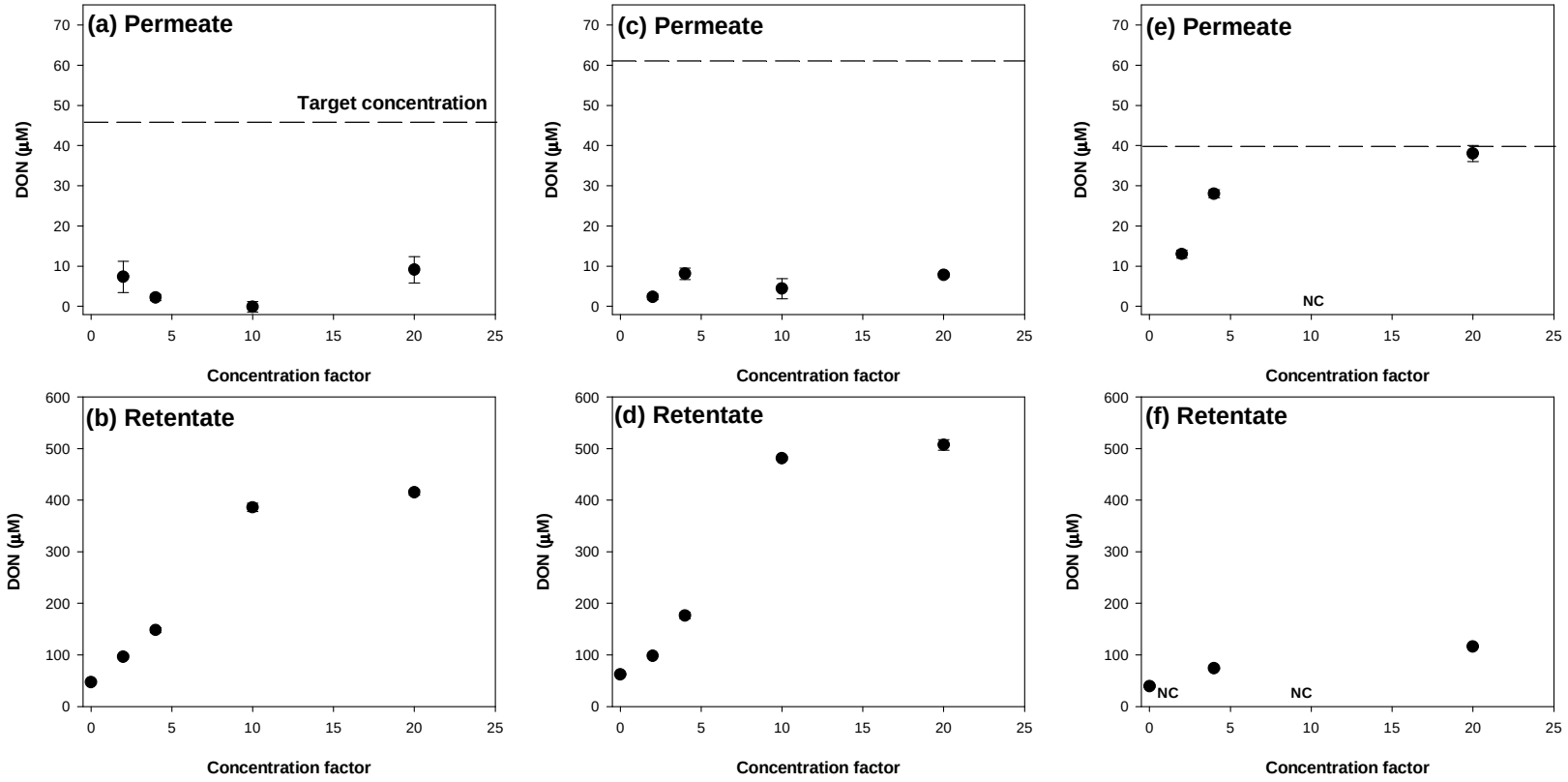


Figure 5.2. DON calibration of 1 kDa ultrafiltration filter using albumin (66 kDa), cytochrome C (12 kDa) and vitamin B-12 (1.33 kDa). The dotted line indicates the initial concentration of the macromolecule solution prior to ultrafiltration. NC indicates not collected. Where no errors are shown, standard deviations (n = 3 or 4) are smaller than the size of the symbol.

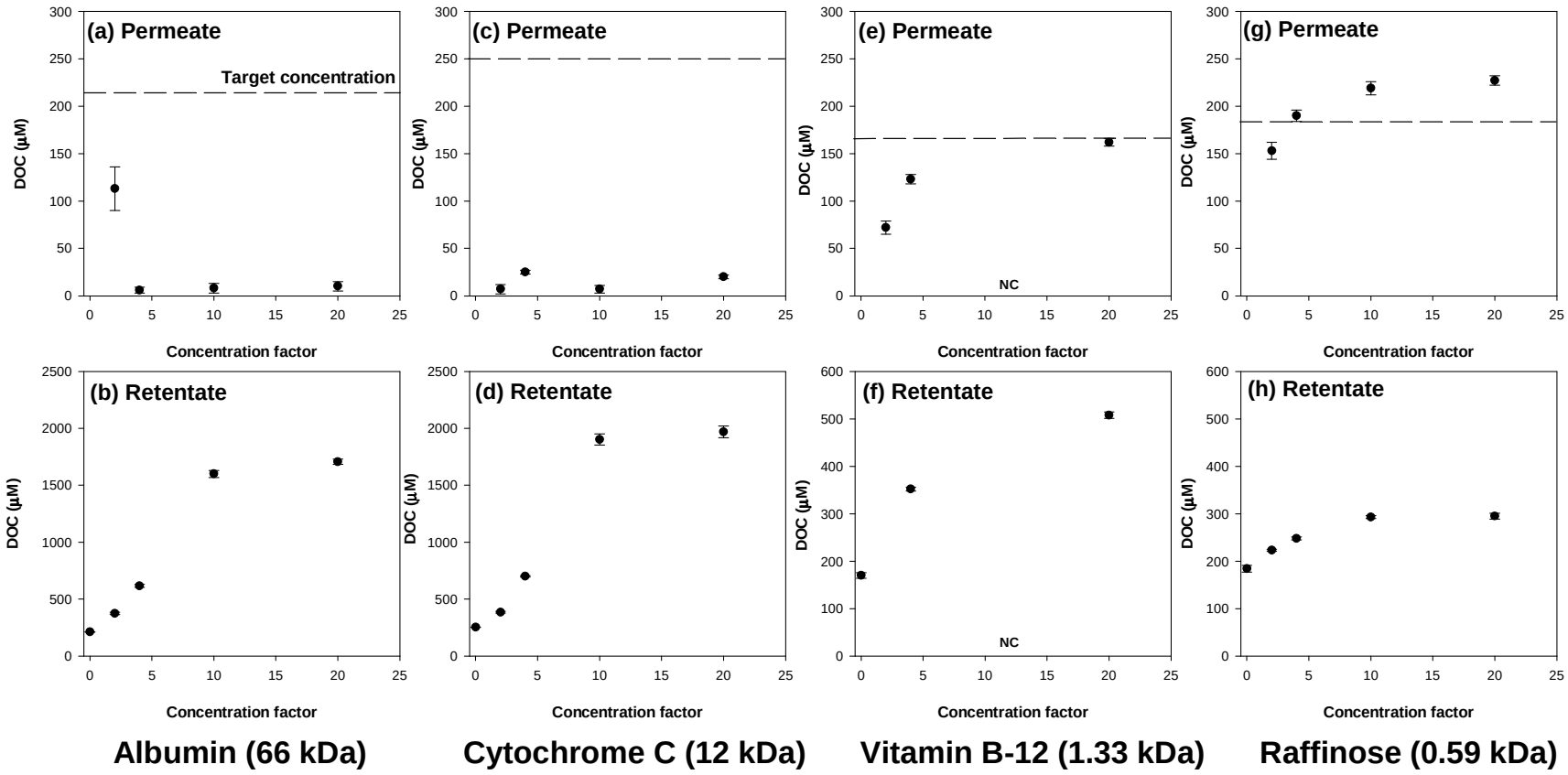


Figure 5.3. DOC calibration of 1 kDa ultrafiltration filter using albumin (66 kDa), cytochrome C (12 kDa), vitamin B-12 (1.33 kDa) and raffinose (0.59 kDa). The dotted line indicates the initial concentration of the macromolecule solution prior to ultrafiltration. NC indicates not collected. Where no errors are shown, standard deviations (n = 3 or 4) are smaller than the size of the symbol.

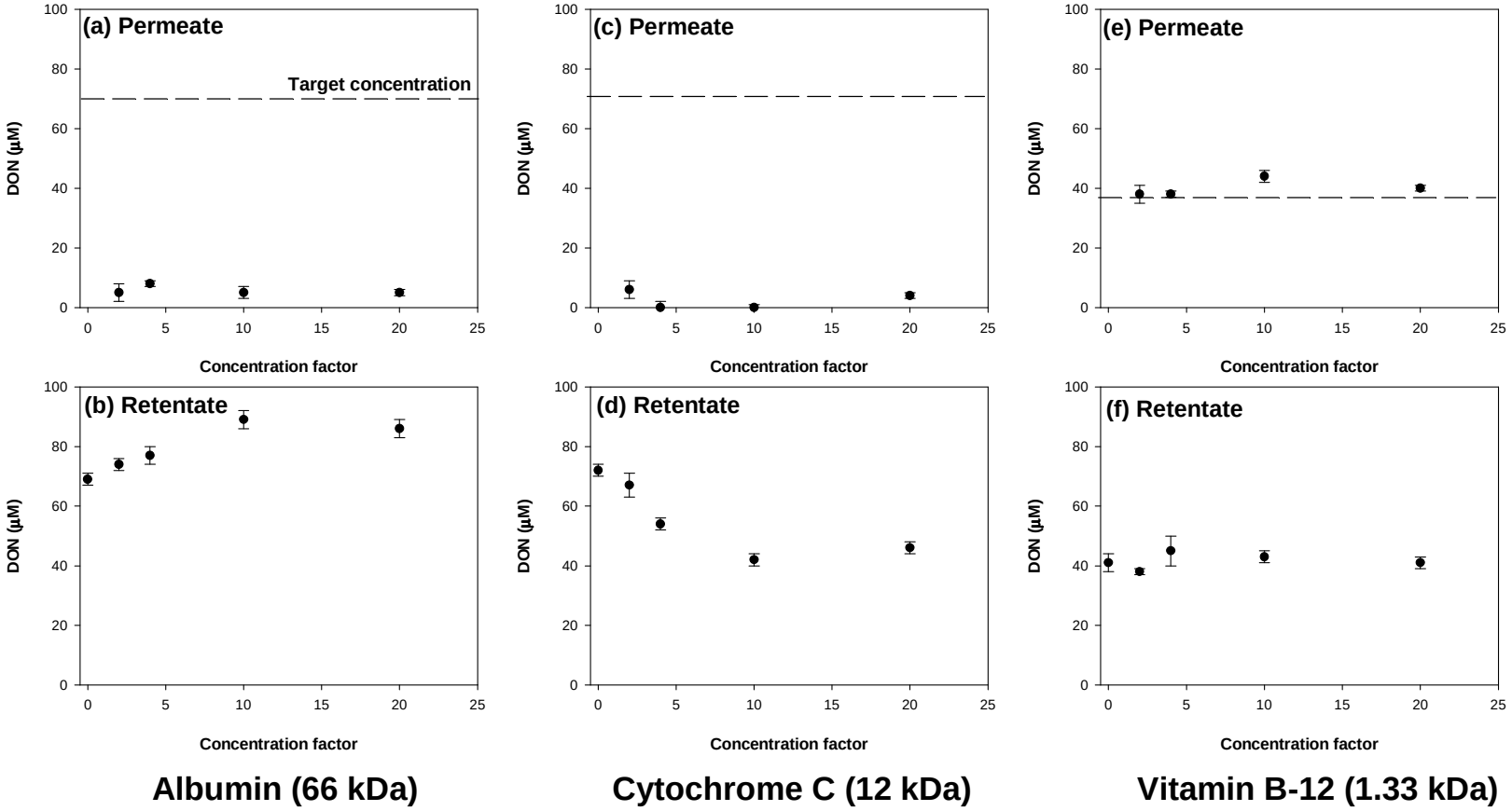


Figure 5.4. DON calibration of 30 kDa ultrafiltration filter using albumin (66 kDa), cytochrome C (12 kDa) and vitamin B-12 (1.33 kDa). The dotted line indicates the initial concentration of the macromolecule solution prior to ultrafiltration. NC indicates not collected. Where no errors are shown, standard deviations (n = 3 or 4) are smaller than the size of the symbol.

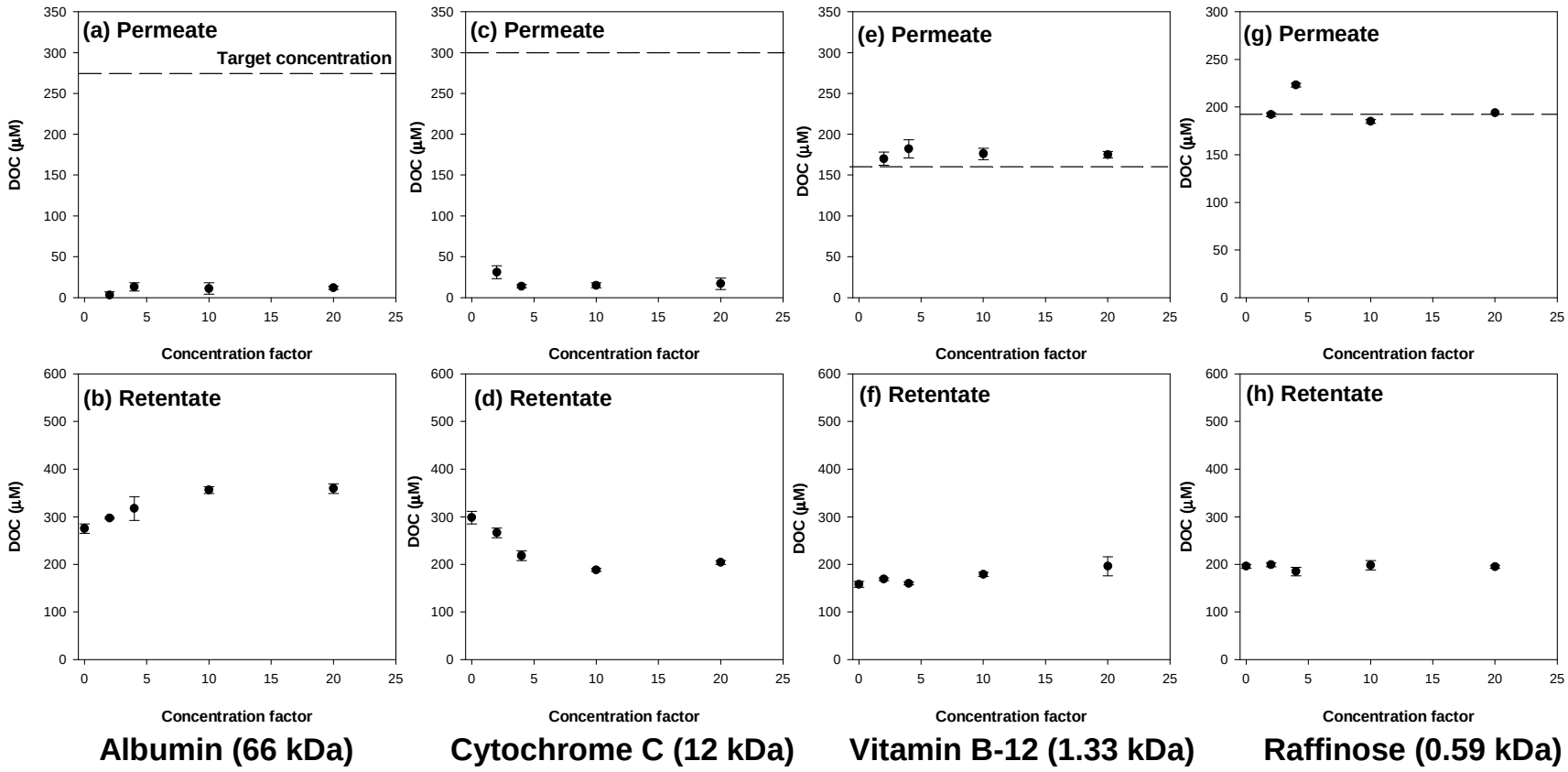


Figure 5.5. DOC calibration of 30 kDa ultrafiltration filter using albumin (66 kDa), cytochrome C (12 kDa), vitamin B-12 (1.33 kDa) and raffinose (0.59 kDa). The dotted line indicates the initial concentration of the macromolecule solution prior to ultrafiltration. NC indicates not collected. Where no errors are shown, standard deviations (n = 3 or 4) are smaller than the size of the symbol.

The data presented in Figure 5.2 to Figure 5.5 are summarised in Table 5.2. The retention characteristics of the filter were assessed at each NMW using a retention coefficient (Equation 5.2), to assess the concentration of the permeate relative to the concentration of the initial solution. The 1 kDa filter had a high retention coefficient for the two largest macromolecules which had NMWs of 12 and 66 kDa. The retention of vitamin B-12 by the 1 kDa filter was negligible, even though the NMW was 1.33, which was higher than that of the 1 kDa filter. As vitamin B-12 was able to pass through the filter into the permeate, this suggests that 1 kDa was an underestimate of the actual NMW cut-off of the filter. The smaller molecular size of the raffinose molecule meant that it had no difficulty passing through the 1 kDa filter.

Table 5.2. Retention coefficients for albumin, cytochrome C, vitamin B-12 and raffinose when pumped through a Prep/Scale™ TFF ultrafiltration cartridge. CF is concentration factor (Equation 5.1) and R is the retention coefficient (Equation 5.2). Raffinose contains no nitrogen.

	NMW (kDa)	DON			DOC		
		Concentration (μM)	CF	R (%)	Concentration (μM)	CF	R (%)
1 kDa filter							
Albumin	66	47	20	81	193	20	100
Cytochrome C	12	62	20	87	253	20	92
Vitamin B-12	1.33	40	20	< 1	175	20	5
Raffinose	0.59	-	-	-	184	20	0
30 kDa filter							
Albumin	66	68	20	93	275	20	96
Cytochrome C	12	72	20	94	298	20	94
Vitamin B-12	1.33	41	20	< 1	158	20	0
Raffinose	0.59	-	-	-	196	20	< 1

The retention coefficient of the 30 kDa filter using DON was 93 – 94 % when albumin (NMW 66 kDa) and cytochrome C (NMW 12 kDa) were used as standards. This was not surprising for albumin, which had a NMW of 66 kDa, which was greater than twice the size of the 30 kDa filter. However, cytochrome C had a NMW of 12 and appeared to be

retained by the 30 kDa filter just as efficiently as the albumin. This suggested that the 30 kDa filter had a lower NMW cut-off than suggested by the manufacturers (i.e. < 12 kDa). Vitamin B-12 had retention coefficients of <1 %, it seems that the majority of these macromolecules were able to pass through the 30 kDa filter into the permeate fraction.

A graphical representation of the retention coefficients from Table 5.2 is presented in Figure 5.6. Retention coefficients for the 1 kDa filter show a sharp increase between the 1.33 and 12 kDa NMW cut-offs (Figure 5.6a). This increased further for DOC and decreased slightly for DON at the 66 kDa NMW. The DON retention coefficient was consistently lower than the DOC, particularly at the higher NMW cut-offs. The 30 kDa filter showed a pattern, with carbon again having the highest retention coefficient at the highest NMW cut-off. Again the nitrogen retention coefficient decreased slightly with the 66 kDa molecules whereas the carbon increased marginally. The reason why a higher retention coefficient was observed for DOC relative to DON is unclear. Although the retention coefficient for DOC was consistently higher, the difference was more distinct from the 1 kDa than 30 kDa filter, indicating that the 1 kDa cut-off was less clearly defined using a combination of DOC and DON than the 30 kDa filter cut-off.

It cannot be assumed, therefore, that the NMW cut-off indicated by the manufacturers are exact for a specific ultrafiltration cartridge. It is therefore important to carry out these calibration experiments with a range of macromolecules to characterise fully the filters. The conclusion from these experiments is that the 1 kDa filter actually has a cut-off >1.33 kDa and the 30 kDa has a cut-off <12 kDa. Further characterisation would be required to define these further. As it is not possible to define the filter cut-offs more precisely, procedural consistency has been obtained therefore by using the same filters throughout the experiments, making the approach repeatable. For the remainder of this study it will be assumed that the two filters have clearly separate NMW cut-offs between 1.33 and 12 kDa, therefore enabling the separation of low (LMW) <1 kDa, high (HMW) 1 – 30 kDa and very high (VHMW) >30 kDa molecular weight fractions of DOM.

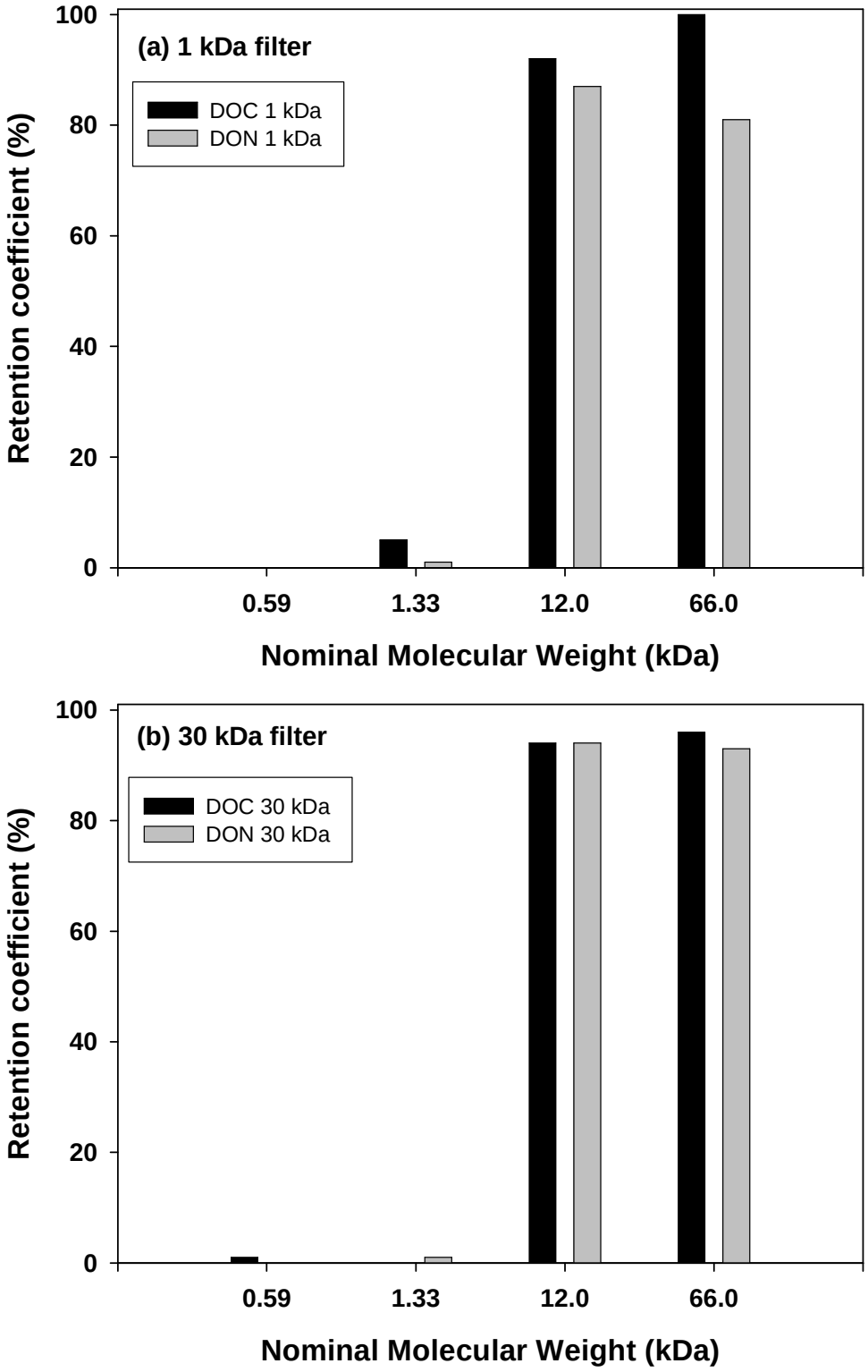


Figure 5.6. Calibration of (a) 1 kDa and (b) 30 kDa Prep/Scale™ –TFF filter cartridges using albumin (66 kDa), cytochrome C (12 kDa), vitamin B-12 (1.33 kDa) and raffinose (0.59 kDa). The retention coefficient indicates the permeate concentration as a percentage of the initial concentration in the initial solution.

5.2.2 Ultrafiltration and incubation procedure

A series of acid washed polycarbonate carbuoys were filled on 20th November 2003, 4th December 2003, 14th, 22nd and 29th January 2004 with water from the freshwater site on the River Test immediately upstream of the tidal limit (site 4; see Appendix H). These were returned to the laboratory for ultrafiltration and additionally, the 3 samples collected in January 2004 were prepared for bacterial bioassay experiments. Initially a subsample of 100 ml was removed to a separate glass bottle. This was filtered through a 2 µm polycarbonate filter to remove detrital material and larger grazers then used as the bacterial inoculum after filtration (Tranvik, 1998). The river water was filtered using a Millipore all glass filtration system and vacuum pump. The GF/F filters (47 mm) had been combusted at 500 ^{o}C for 4 hours and were replaced when the filter became blocked. A second filtration was then carried out using 47 mm 0.2 µm (Isopore membrane) filters to remove bacteria from the water (Benner, 1991).

The filtrate from 0.2 µm filtration was sub-sampled to determine the original nitrate and TDN concentrations. An aliquot of 10 ml was removed to a plastic capped vial for nitrate analysis and 50 ml to a pre-combusted (550 ^{o}C for 4 hours) 100 ml glass bottle for TDN and DOC analysis. Finally, 90 ml of the filtrate was added to each of the three separate conical polycarbonate incubation flasks. These flasks contained the >30 kDa (VHMW), <30 kDa (HMW and LMW) and <1 kDa (LMW) fractions. To each flask 10 ml of the bacterial inoculum (unfiltered water) was added and agitated (Tranvik, 1998). The samples were stored in a dark, sealed box in a temperature-regulated room at 18 ^{o}C.

The incubation flasks were sampled on days 0, 1, 3 and 6 to determine the bacterial abundance. The technique used to enumerate the bacteria was DAPI staining and epifluorescent microscopy (Porter and Feig, 1980). A glass filtration system was assembled with a 0.45 µm, 25 mm white cellulose acetate filter beneath a 0.2 µm black polycarbonate filter and the filters were dampened using 0.2 µm filtered milli-Q water. Samples were removed from the dark and 1 ml pipetted into the glass filtration system. Formalin (50 µl of 37 % which had been filtered through a 0.2 µm filter) was added to the sub-sample and agitated. Finally 100 µl of 1,2-phthalic dicarboxaldehyde (DAPI) stain was added to the filtration system. The solution was mixed and left to stain the bacteria for 10 minutes, after which the stained solution was drawn through the filter using a hand

vacuum pump. The ratio of incubation sample volume to formalin and DAPI stain remained identical for all samples. However, when the bacterial numbers were low, the volume of incubation sample was often increased to 2 ml to obtain a more precise bacterial count.

The black filter was mounted using immersion oil on a large microscope slide and cover slip. Three DAPI stained filters were produced from each incubation flask on each sampling date. A Fluorosepec Leitz microscope fitted with a 50 W mercury vapour burner UV lamp was used to count the bacteria in 10 fields of view at 1000x magnification. The mean number of cells in a field of view was used to determine the bacterial abundance (Equation 5.3).

$$\text{Bacterial abundance (cells ml}^{-1}) = C \text{ x} \left(\frac{f}{f} \right) \text{x} \left(\frac{1}{v} \right) \qquad \text{(Equation 5.3)}$$

C = cells per field of view
f = filter area (mm^2)
f = field area (mm^2)
v = the volume filtered (ml)

The remaining 0.2 µm filtered river water was processed through a series of Millipore Prep/Scale TFF™ spiral wound filters contained within cartridges at the nominal molecular size cut-offs of 1 and 30 kDa. Cartridges were stored with 0.1 M NaOH and in the filter holder (or alternatively the fridge for longer-term storage). The storage solution was removed and the cartridges flushed with volumes of Milli-Q water in excess of 9 L until a pH > 5 was obtained. Cleaning procedures were verified by calculating the normalised water permeability prior to each use of the cartridge for filtration. Blanks were measured from permeate samples collected after filtering UV irradiated Milli-Q water. These gave concentrations of DON and DOC <10 µM. The 0.2 µm filtered river sample was initially processed through the 30 kDa filter, removing and concentrating the molecules >30 kDa in the retentate and thereby producing the VHMW fraction. The permeate contained the DOM <30 kDa and was therefore sub-sampled for nitrate, DOC and DON analyses and 90 ml placed in each of three conical incubation flasks. The incubation flasks containing the 30 kDa permeate were composed of LMW and HMW DOM. The bacterial inoculation, incubation and cell counting was identical to that described above for the 0.2 µm filtrate.

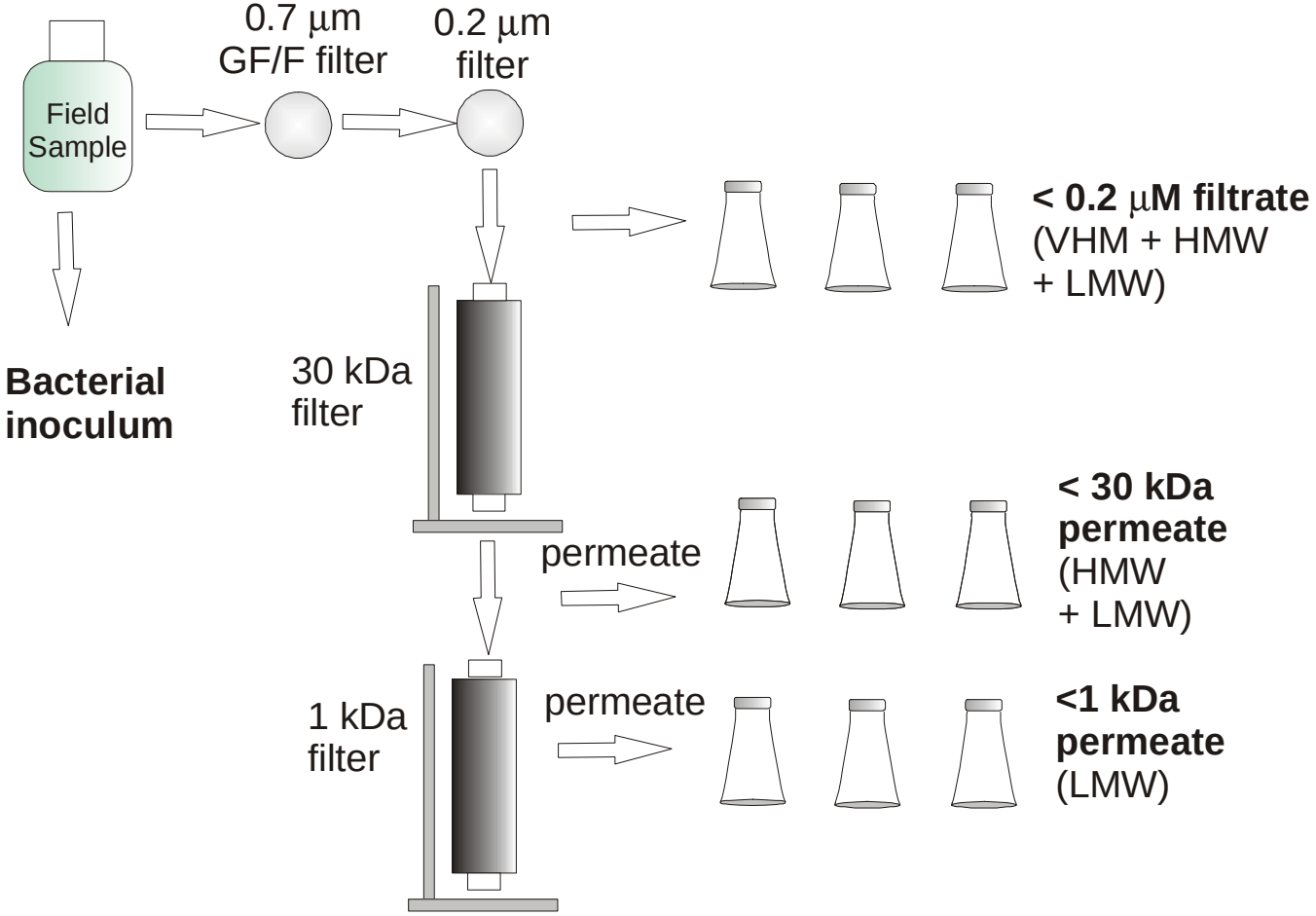


Figure 5.7. Filtration procedure for preparing bacterial bioassays

The remaining 30 kDa permeate was passed through the 1 kDa filter and the permeate collected and treated in an identical manner to the other fractions. The permeate from the 1 kDa filter contained LMW DOM <1 kDa. The retentate contained a concentrated fraction of HMW DOM. A schematic diagram of the first steps of the procedure is presented in Figure 5.7.

5.3 RESULTS

5.3.1 Environmental sampling conditions November 2003 - January 2004

Water was collected on 20th November 2003, 4th December 2003, 14th, 22nd and 29th January 2004 respectively and returned to the laboratory for subsequent ultrafiltration and incubation of bacteria over 6 days. The environmental parameters at the time of sample collection are listed in Table 5.3. The conductivity of the river water on 14th January 2004 was lower than the other sampling dates. During the experiment the range of flows measured at Testwood gauging station at (Appendix H) 15 minute intervals was between 3 and 33 m^{-3} s^{-1} (Figure 5.8). Therefore the river samples were collected from a wide range of flow conditions. The flow was fairly low in November and December 2003 with only a couple of peak flows probably caused by precipitation events. The flow during January 2004 was much higher, although between 19th and 22nd January several rapid changes in flow were observed which resulted in a wide range of flow being recorded during the 22nd January. It is also worth noting that the water temperature was much lower on the last sampling date, which was probably caused by the cold weather; ice and snow on the ground.

Table 5.3. Environmental parameters measured during sample collection at site 4 from November 2003 to January 2004. * ice and snow on the ground

	Sampling date				
	20/11/03	**04/12/03**	**14/01/04**	**22/01/04**	**29/01/04***
Time	09:00	09:30	10:30	09:00	09:00
Salinity	0	0	0	0	0
Temperature (^{o}C)	11.4	9.0	8.1	8.8	4.2
Conductivity (µS cm^{-1})	574	566	381	508	585
River flow (m^3s^{-1})	3	9	17	11.5	11.5

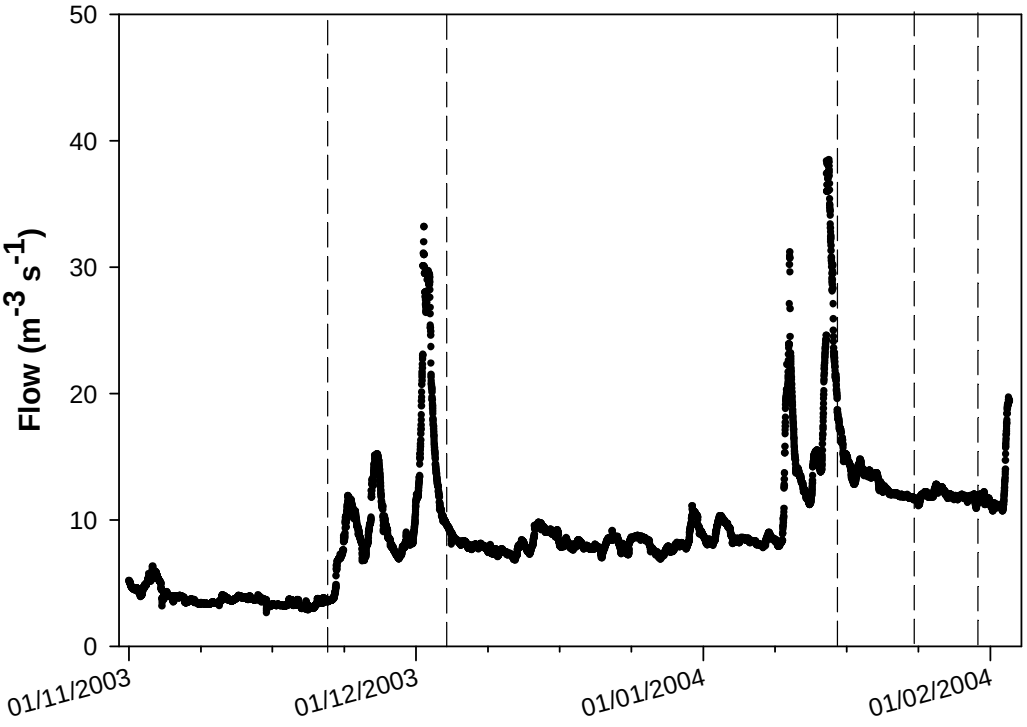


Figure 5.8. River flow at Testwood (SU 3539 1526) at 15 minute intervals from November 2003 to January 2004. Dotted lines indicate collection of river water for incubation of samples on 20th November 2003 (3 m^{-3} s^{-1}), 4th December 2003 (9 m^{-3} s^{-1}), 14th (17 m^{-3} s^{-1}), 22nd (11.5 m^{-3} s^{-1}) and 29th January 2004 (11.5 m^{-3} s^{-1}).

5.3.2 Ultrafiltration

Ultrafiltration permeate nutrient concentrations Samples for determination of nitrate, dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) were collected during the course of the ultrafiltration process. Triplicate samples were collected from the 0.2 µM filtered water (VHMW + HMW + LMW) prior to ultrafiltration as well as from the permeate fraction from the 30 kDa (HMW + LMW) and 1 kDa (LMW) filter (Figure 5.9a–o).

The nitrate concentration in the River Test at site 4 on 20th November 2003 was 443 µM (Figure 5.9a). Similar concentrations were measured in the permeate from the 30 kDa filter, although concentrations were lowest in the 1 kDa permeate. The observation of a decrease in the nitrate concentration as the sample passes through the 1 kDa filter cartridge will be discussed later in this section. The concentration of DON in the water sample collected on 20th November 2003 was 17 µM (Figure 5.9b). There was little change when filtered through the 30 kDa filter, but there was a concentration decrease to 6 µM when filtered through the 1 kDa filter. A similar pattern was observed from the concentration of DOC, with 152 µM being recorded in the original river water (Figure 5.9c). After filtration through the 30 kDa filter, 147 µM DOC was measured. The final filtration through the 1 kDa membrane resulted in a permeate with the lowest concentration of DOC measuring 125 µM.

A nitrate concentration of 395 µM was recorded in the original river sample collected on 4th December 2003 (Figure 5.9d). This was only 2 µM higher than the concentration measured in the 1 kDa permeate. A slightly higher concentration was recorded in the 30 kDa permeate (403 µM). DON concentrations decreased as the initial river water was passed through the ultrafiltration filters (Figure 5.9e). The initial DON concentration was 43 µM, of which 33 µM passed through the 30 kDa filter and only 17 µM was of a smaller molecular size than 1 kDa. The DOC showed a similar trend with 27 µM difference being measured between the initial (374 µM) and 30 kDa permeate (347 µM) (Figure 5.9f). Less than half the 30 kDa DOC concentration was recorded in the 1 kDa permeate (156 µM).

On 14th January 2004 the nitrate concentrations in the water sample were 307 µM (Figure 5.9g). Permeate from the 30 kDa filter contained a slightly higher nitrate concentration of

312 µM; although the lowest concentrations of nitrate were recorded in the 1 kDa permeate. The concentration of DON on 14th January determined from measurements of total dissolved nitrogen (TDN) and nitrate was 27 µM (Figure 5.9h). The lowest concentration of DON was recorded in the 1 kDa permeate. The DON in the 0.2 µM filtered, 30 kDa and 1 kDa permeate was 27 µM, 22 µM and 20 µM respectively. A similar trend was seen in the concentrations of DOC but a larger difference of 183 µM was observed between the concentrations in the 30 and 1 kDa permeates. The concentrations of DOC in the 0.2 µM filtered, 30 kDa and 1 kDa permeate were 607 µM, 594 µM and 411 µM respectively (Figure 5.9i).

Nitrate concentrations measured on 22nd January 2004 were much higher than seen the previous week (Figure 5.9j). The nitrate concentration in the original river water that passed through the 0.2 µM filter was 522 µM. Permeate from the 30 kDa filter which contained HMW and LMW material had a nitrate concentration of 483 µM. The concentration in the 1 kDa permeate containing LMW material was 23 µM lower than the 30 kDa permeate. Concentrations of DON in the sample collected on 22nd January 2004 showed a high degree of variation with large errors from the compounded standard deviation associated with the nitrate and TDN measurements (Figure 5.9k). The DON was highest in the 30 kDa permeate and similar concentrations were recorded in the 0.2 µm filtered and 1 kDa permeate samples. All DON values were derived by difference and a negative value of DON was recorded in the 0.2 µm and 1 kDa permeate samples collected on 22nd September. This was an analytical problem that occurred with low concentrations of DON that were close to the detection limit as the derived DON value was dependant on the accuracy of the TDN and nitrate measurements (Hansell, 1993; Hopkinson *et al.*, 1993; Hedges *et al.*, 1994a; Bronk *et al.*, 2000). DOC concentrations of 188 µM were recorded after the water sample passed through the 0.2 µM filter (Figure 5.9l). A lower concentration of 157 µM was recorded in the permeate from the 30 kDa filter. There was a DOC concentration of 68 kDa in the LMW material from the 1 kDa filter.

Concentrations of nitrate, DOC and DON in the water sample collected on 29th January 2004 were similar to those measured a week earlier. The nitrate concentrations in the 0.2 µm filtered and 30 kDa permeate were identical, with concentrations of 512 µM (Figure 5.9m). The nitrate concentration in permeate fraction from the 1 kDa filter was much lower at 455 µM. DON in the water sample was negligible with no apparent variation

between the different molecular size fractions (Figure 5.9n). The concentrations of DOC were comparable with those measured on 22nd January, and were generally less than 200 µM (Figure 5.9o). The permeate from the 1 kDa filter containing LMW material contained 50 % of the total DOC in the 0.2 µm filtered sample.

Retention of nitrate by ultrafiltration cartridges Measurements of nitrate from the permeate fractions were carried out for the determination of DON in river water (ammonium concentrations were considered negligible). Nitrate has a simple, small molecular structure and therefore was expected to be unaffected by the ultrafiltration process. However, clear decreases in nitrate concentrations were observed particularly after the final filtration through the smallest 1 kDa filter (Figure 5.10). This decrease between nitrate concentrations in the 30 and 1 kDa permeate as well as between the 0.2 µm filtered and 1 kDa permeate was consistent on all sampling dates. A slight increase was observed on some sampling dates indicating increased concentrations of nitrate in the 30 kDa permeate. This was usually a negligible increase that was within the errors of the analysis.

Characterisation of molecular size fractions The measurement of nitrate, TDN and DOC in the original river water and the water that passed through the 30 and 1 kDa filters can be used to determine the concentration of these nutrients in each of the molecular size fractions. The 30 kDa filter removed molecules greater than 30 kDa therefore leaving permeate only containing LMW and HMW material. The difference between the nitrate, DON and DOC concentrations in the original river water (containing all molecular size fractions) and the 30 kDa permeate gave the concentrations in the very high molecular size fraction (VHMW). Subtraction of the nutrient concentrations in the 1 kDa permeate (containing LMW material) from the 30 kDa permeate (containing HMW and LMW material) gave an indication of the nutrient concentrations in the HMW fraction (>1 kDa and <30 kDa). An assumption was made that nitrate was not that dissimilar in all flasks although a small amount was retained during ultrafiltration.

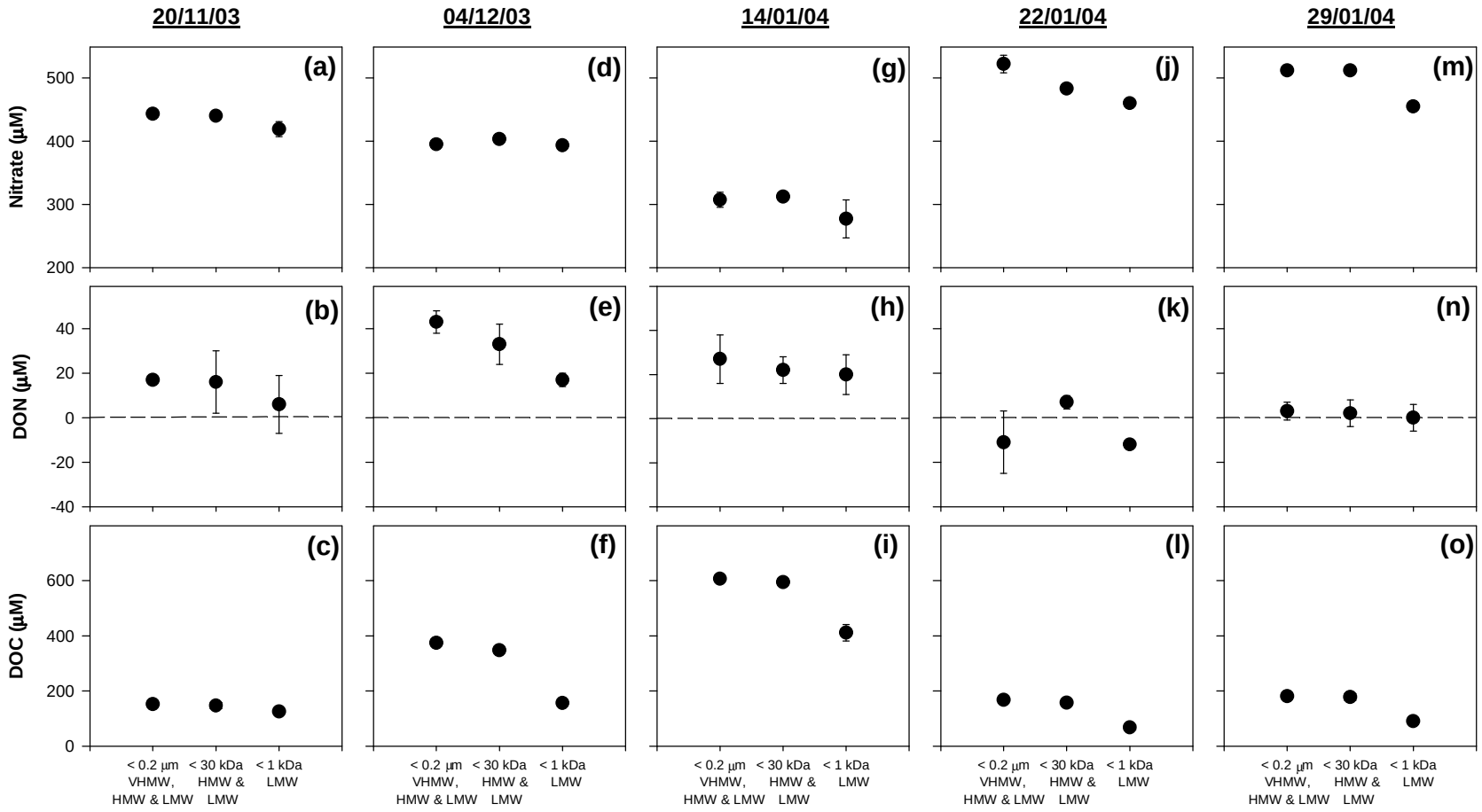


Figure 5.9. Concentrations of nitrate, DON and DOC in ultra-filtered water samples collected at site 4 on 20th November 2003, 4th December 2003, 14th, 22nd and 29th January 2004. Where no errors are shown, standard deviations are smaller than the size of the symbol.

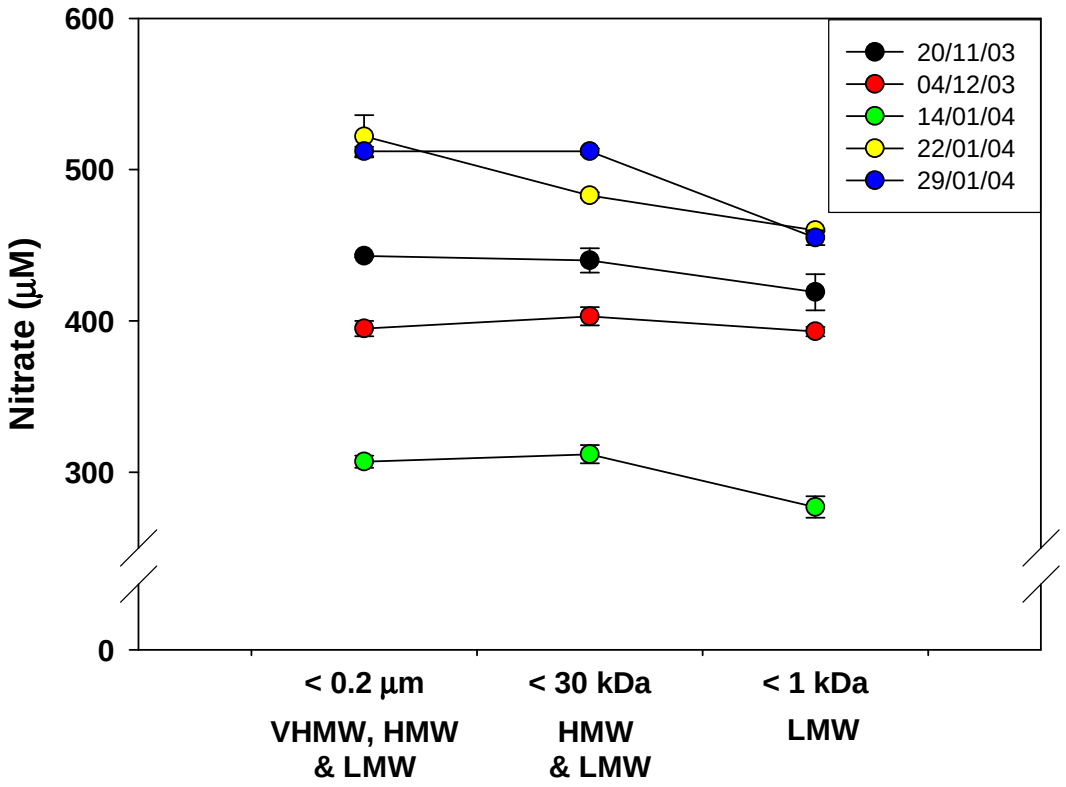


Figure 5.10. Change in nitrate concentrations between the 0.2 µm filtered fraction and 30 kDa and 1 kDa permeates. Samples were collected on 20th November 2003, 4th December 2003, 14th, 22nd and 29th January 2004. Errors represent one standard deviation of 3 replicate samples, each analysed up to three times.

The nutrient concentrations in molecular size fractions of the samples collected from November 2003 to January 2004 are presented in Figure 5.11a – j. The concentrations in each molecular weight fraction are shown in Table 5.4 and Table 5.5 as a percentage of the total DON or DOC. The DON concentration at site 4 on 20th November 2003 was 17 µM (Figure 5.11a), of which 10 µM (60 %) was composed of HMW material. The second largest fraction (36 %) was the LMW material comprising 6 µM, and the smallest component, comprising only 3 % of the total was 1 µM VHMW DON. Large errors were associated with the determination of DON in these fractions, which were a product of subtracting large values for nitrate and TDN. The total concentration of DOC was 152 µM in the river water collected at site 4 on 20th November 2003 (Figure 5.11b). The smallest fraction of this was 3 % VHMW DOC, which comprised 4 µM. LMW material was the largest fraction of DOC (82 %), with a much smaller proportion of the total (15 %) being composed of HMW material.

Table 5.4. Percentage composition of DON comprised of LMW (<1 kDa), HMW (>1 kDa and <30 kDa) and VHMW (> 30 kDa). Lack of data for 22nd January 2004 reflects a lack of data not an absence of DON.

	LMW %	HMW %	VHMW %
20/11/03	36	60	3
04/12/03	40	37	23
14/01/04	74	7	19
22/01/04	-	-	-
29/01/04	0	50	50

Table 5.5. Percentage composition of DOC comprised of LMW (<1 kDa), HMW (>1 kDa and <30 kDa) and VHMW (> 30 kDa).

	LMW %	HMW %	VHMW %
20/11/03	82	15	3
04/12/03	42	51	7
14/01/04	68	30	2
22/01/04	43	51	7
29/01/04	51	48	1

On 4th December there was 43 µM DON in the river water at site 4. This was composed of almost equal concentrations of 40 % HMW (16 µM) and 37 % LMW (17 µM) DON (Figure 5.11c). The remaining 23 % (10 µM) was composed of VHMW DON. The DOC

concentration on 4th December 2003 was 374 µM. Some 51 % was composed of HMW (192 µM) and 42 % LMW material (156 µM). The VHMW DOC made up the smallest fraction comprising only 26 µM which was 7 % of the total (Figure 5.11d).

The DON concentration at site 4 on 14th January was 27 µM (Figure 5.11e). This was composed of 20 µM LMW which was the largest fraction (74 %), 5 µM VHMW (19 %) and the smallest fraction (7 %) was HMW composed of 2 µ M DON. The DOC concentration in the sample collected on 14th January 2004 was 607 µM, of which 411 µm (68 %) was composed of LMW material (Figure 5.11f). Similar to the molecular size composition of the DON, the highest concentration was in this LMW fraction. The HMW fraction contained 183 µM DOC (30 %). The smallest molecular size fraction of DOC (2 %) was the VHMW material; this had a concentration of 14 µM.

Molecular size fractions of the DON in the sample collected on 22nd January 2004 could not be properly determined due to the error of the calculation method; the concentrations of DON appeared to be negative (Figure 5.11g). Although analyses were repeated as indicated in Chapter 2 the concentrations for DON appeared to remain negative. The concentration of DOC in the water sample collected at site 4 was 168 µ M (Figure 5.11h). The composition of the DOC when separated according to molecular size fractions was different to the sample collected on 14th January 2004. The highest concentration of DOC (89 µM) was measured in the HMW fraction (51 %) and the lowest concentration was in the VHMW fraction (7 %).

The DON in the river sample collected on 29th January was also negligible and little difference was apparent in the concentrations of DON in the HMW and VHMW size fractions (Figure 5.11i). The total concentration of DOC in the sample collected on 29th January was 181 µM. This was similar to that collected a week earlier, and a third of that measured on 14th January (Figure 5.11j). The lowest concentration of DOC was recorded in the VHMW fraction (1 %) and the concentrations in the HMW (48 %) and LMW (51 %) fractions were similar at 87 and 93 µM respectively.

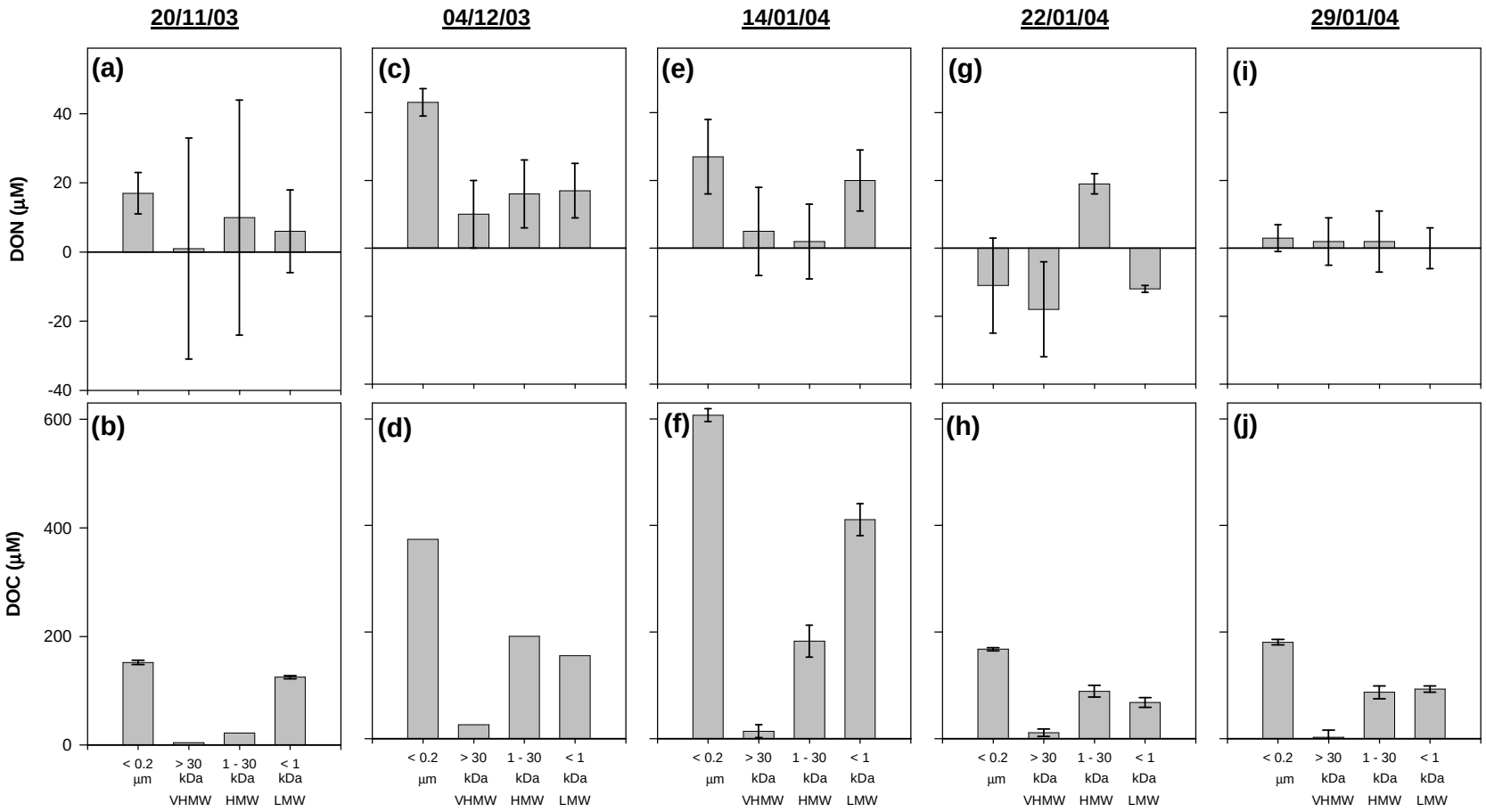


Figure 5.11 DON and DOC concentrations in each molecular size fraction VHMW (> 30 kDa), HMW (> 1 kDa and <30 kDa) and LMW (<1 kDa). Error bars indicate compounded standard deviation of up to four replicates.

5.3.3 Bacterial Bioassay incubations

Bacterial bioassays commenced on 14th, 22nd and 29th January 2004 using molecular size fractionated river water. Bacterial counts were carried out on days 0, 1, 3 and 6 and one DAPI stained filter was produced per day from each of the nine incubation flasks (triplicate flasks of 0.2 µm, 30 kDa and 1 kDa filtered river water). The mean bacterial abundance from ten fields of view on each of the DAPI stained filters were converted to cells ml^{-1} using equation 5.3. It was observed that the longer the flasks were incubated, the more similarly the bacteria behaved, therefore data from days 0 to 3 are presented for each set of flasks (Figure 5.12). Lines were used to link the bacterial counts from the same set of incubation flasks throughout the incubation period.

The original abundance of natural bacteria in the river water inoculum, prior to ultrafiltration, changed markedly between sampling dates. On 14th January 1.2×10^6 cells ml^{-1} were recorded which was much higher than the bacterial counts on the other two sampling dates which were ten fold lower. Bacterial abundance for 22nd and 29th January was 1.5×10^5 and 1.1×10^5 respectively. The highest original bacterial abundance coincided with the highest river flow at the time of sampling on 14th January 2003.

The first bacterial bioassay commenced on 14th January and had a rapid increase in bacterial abundance between days 0 and 1, when the peak abundance was recorded (Figure 5.12a). The abundance had declined by day 3 to similar levels as seen on day 0. The only flask to grow bacteria closest to the original abundance contained the 1 kDa permeate.

There was good agreement between bacterial abundances for the three molecular size fractions, although some patterns were observed. The lowest bacterial abundances on each day of the incubation were recorded in the flasks containing the 30 kDa permeate fraction. There was, however, a large difference in the bacterial numbers between the replicate flasks, particularly on day 3. The highest and also the widest range of bacterial abundances on days 1 and 3 were recorded for the 1 kDa permeate. There was considerable overlap between the three molecular size fractions on all days.

The second incubation, commencing on 22nd January, behaved very differently to that on 14th January (Figure 5.12b). The majority of incubation flasks showed a decline in

bacterial abundance to virtually no bacteria on day 1, and then the bacterial abundance proceeded to increase gradually from day 1 to a peak on day 6. None of the nine flasks during the 6 day incubation grew bacteria back to the initial abundance in the inoculum.

There was a high degree of consistency between the bacterial abundances measured in replicate flasks throughout the incubation. On days 0 and 1 it was not possible to differentiate clearly which molecular size fractions contained the highest bacterial abundances. The separation became clearer by day 3 and a particularly high bacterial abundance was recorded in the 0.2 µm replicate 3 flask, although this was not replicated in the two other incubation flasks of the same molecular size fraction.

The last bacterial bioassay using water collected on 29th January 2004 was once again very different to the other two sampling dates (Figure 5.12c). There was an increase in the bacterial abundance in the 0.2 and 1 kDa flasks from between days 0 and 1, although a slight decrease was observed in the 30 kDa flasks. By day 3 a rapid increase in bacterial abundance had been recorded to a peak bacterial abundance in all flasks, which had not been observed in the other two incubation experiments.

The third bacterial bioassay had the greatest degree of variation between the replicate bacterial abundances from the same molecular size fractions. The bacterial abundances on day 0 had a high degree of agreement despite a large error associated with bacterial abundance in individual flasks (Figure 5.12c). However, by day 1 the greatest bacterial abundances were recorded in the 0.2 µm filtered river water, this was also observed on day 3. There was no clear separation between these and the flasks containing the other two molecular size fractions, as there was a high degree of similarity between the range of bacterial abundances in the replicate flasks containing the 1 and 30 kDa permeate.

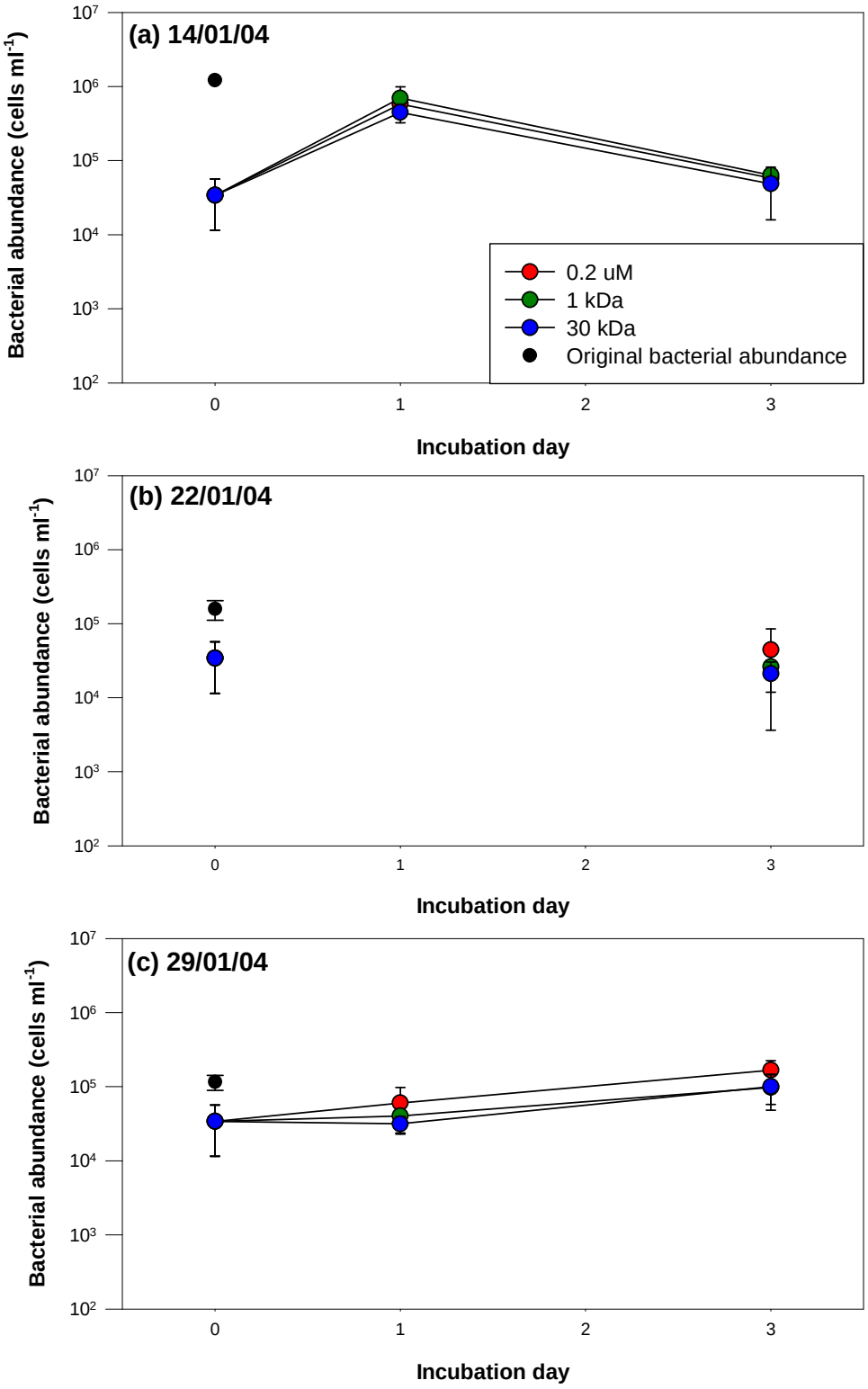


Figure 5.12. Bacterial counts on days 0, 1 and 3 of incubation experiments started on (a) 14th, (b) 22nd and (c) 19th January 2004. Error bars represent one standard deviation of bacterial counts in 10 fields of view on a DAPI stained slide from three replicate vessels. The dotted line represents the original bacterial abundance in the natural river water inoculum.

5.4 DISCUSSION

Quantification of the proportion of freshwater DOM that is bioavailable for biological production is necessary to produce accurate budgets for nitrogen entering estuaries that contribute to biological production. The use solely of DIN in the past has perhaps underestimated the potential nitrogen inputs to these dynamic systems (Seitzinger and Sanders, 1997b). Although previous work has focused on DOC (Carlson *et al.*, 1985; Cauwet, 2002) and since it is not possible to separate pools of DOC and DON, these have been considered together in terms of the bioavailability of DOM. Bacterial bioassays were used to assess bioavailability as DOM is considered an important carbon and energy source for bacteria (Fuhrman, 1992; Benner *et al.*, 1997; Goldman and Dennett, 2000). The microbial degradation of DOM is also considered one of the most important removal mechanisms in river and estuarine systems (Wiegner and Seitzinger, 2001), for which rates of biological removal are greater than those from photochemical degradation of DOM (Wiegner and Seitzinger, 2001; Benner, 2002). The majority of riverine bioavailability work has focused on specific chemical groups such as the dominance of humic substances in riverine DOM (Carlsson and Granèli, 1993; Cauwet, 2002). Meybeck (1993) states that the chemical characterisation of riverine DOM using size fractionation of organic matter has been overlooked.

5.4.1 Defining ultrafiltration cartridge NMW cut-off

To define clearly the size of molecules that an ultrafiltration cartridge is able to retain it is necessary to determine the molecular size cut-off. The cartridges were selected with nominal molecular weight cut-offs to coincide with the definitions of low, high and very high molecular weight fractions. Although 1 kDa is widely accepted as the upper size range for LMW material regardless of the aquatic environment, the differentiation between HMW and VHMW on the basis of a 30 kDa cut-off is only recognised in freshwater and coastal literature (Amon and Benner, 1996).

The ability of a cartridge to retain macromolecules of a specified molecular size was assessed using the retention coefficient of both DOC and DON. These were often in good agreement with a high retention coefficient indicating an inability for a molecule to pass through the membrane into the permeate. The retention coefficient for DON was generally consistently lower than DOC, particularly for the 1 kDa filter. This may be related to the

higher concentrations of DOC prepared in the calibration standards thereby causing a larger concentration gradient across the filter membrane than for DON. Although other factors may be involved such as physio-chemical interactions between macromolecules and the filter, membrane adsorptive losses or breakthrough of material that is greater than the NMW cut-off (Mannino and Harvey, 2000).

The two ultrafiltration cartridges showed a similar retention of macromolecules despite their apparently different nominal molecular weight cut-offs. Vitamin B-12 (1.33 kDa) which should have been close to the cut-off of the 1 kDa membrane, showed a distinct difference in retention between the two filters. The DOC retention of vitamin B-12 was higher for the 1 kDa than 30 kDa filter. Further tests using a macromolecule with a MW between 1.33 and 12 kDa would have shown a clearer distinction between NMW of the two filters. Without this it can be established that the filter with the 1 kDa NMW was not able to retain vitamin B-12 and therefore had a cut-off > 1 kDa. Similarly the filter with the 30 kDa NMW was able to retain cytochrome C and therefore had a lower cut-off 12 kDa.

5.4.2 Nitrate retention

Molecules that are smaller than the molecular weight cut-off of the filter membrane were expected to pass through the filter membrane (Hollibaugh *et al.*, 1991). This meant that all the bacterial bioassays, regardless of DOM content, would have the same concentration of nitrate in both the permeate and retentate. However, a marked decrease in the nitrate concentration was consistently observed after filtration through the 1 kDa membrane. This indicated that nitrate was being retained by the ultrafiltration process. Although this hasn't previously been observed for nitrate, adsorption of molecules on membranes has been observed in other investigations and can result in unrepresentative size fractions (Mannino and Harvey, 2000). Other research has found sulphate, calcium and magnesium retention by ultrafiltration cartridges which could be attributed to electrostatic differences between the ions and the cartridge membrane causing repulsion of the major ions (Guo *et al.*, 2001). The retention of nitrate by the 1 kDa filter may be cause by similar charge differences.

5.4.3 Molecular size fractions of DOM

There is good agreement in the literature that HMW plus VHMW (i.e. >1 kDa) are the dominant molecular size fractions of DOC in freshwater samples (Benner and Hedges, 1993; Hedges *et al.*, 1994b; Amon and Benner, 1996; Perdue and Ritchie, 2003). In contrast there has been little work on the ultrafiltration of DON compounds (Stepanauskas *et al.*, 1999b). The author found only two studies on fractionation of freshwater DON. The first was based on wetlands in Sweden where LMW was found to be the dominant fraction of DON (Stepanauskas *et al.*, 1999b) and the second had similar conclusions from water collected in the Elbe estuary, Germany (Kerner and Spitzy, 2001). Better characterisation of the size fractions of DOM and their chemical composition are needed to determine the bioavailability of these fractions.

There was no consistently higher concentration of DON or DOC in any of the molecular size fractions from the ultrafiltration experiments. LMW was found to be both the dominant DOC and DON fraction on only one sampling date (14th January 2004), when the highest river flow was recorded, although concentrations of DON were only just higher than measured on other sampling dates. It is possible that the chemical composition of river water varies temporally, although these experiments suggest that the changes may be more frequent than on a seasonal basis.

There was also a lack of agreement between the percentage composition of DOC and DON. This suggests that there was a high degree of decoupling between carbon and nitrogen in the DOM. The composition tended to be dominated by either LMW or HMW material on the majority of sampling dates. It was noticeable that the DOC VHMW fraction was < 10 % on all sampling dates, which was in contrast with DON which had a mean VHMW of 24 %.

5.4.4 Bacterial bioavailability

Conventionally LMW was believed to be the most biologically available to bacteria and phytoplankton due to its simple small molecular structure (Saunders, 1976). Recently there has been a change of opinion since LMW was found to comprise only a small proportion of DOM in natural waters. Instead the newer and diagenetically younger HMW DOM appears to be more bioavailable (Amon and Benner, 1996). This was the basis of the size-reactivity continuum model proposed to explain DOM bioavailability (Tranvik,

1990; Benner *et al.*, 1992; Amon and Benner, 1994, 1996; Kerner and Spitzy, 2001). Therefore by identifying the molecular size composition of DOM it should be possible to test the bioavailability of the various fractions (Meybeck, 1993; Kirchman, 1994), at least to a first approximation

The results from these bioassay experiments show an overlap in the bacterial abundances associated with each molecular size fraction. There was no statistical difference between the bacterial growth in the 0.2 μm filtered, LMW (<1 kDa) or LMW plus HMW (<30 kDa) fractions. One possible reason may be the low concentrations of DOC and DON in the original freshwater samples after fractionation using ultrafiltration. This may have resulted in insufficient DOM to support appreciable bacterial growth. Repeating the experiments during the late summer months may give higher concentrations of DON and DOC for ultrafiltration and bacterial bioassay experiments. A possible reason for the low bacterial abundances are that chemical characteristics of DOM have been observed to change downstream (Sun *et al.*, 1997). The compounds in the lower reaches of the Ogeechee River, Georgia (USA) were more degraded than further upstream and had a lower aliphatic carbon content which may reflect a lower bioavailability of DOM (Sun *et al.*, 1997).

The first incubation experiment carried out on 14th January 2004 was the only bacterial incubation to almost reach the original bacterial abundance. This experiment experienced the highest flow conditions suggesting that the higher flow rates result in higher amounts of bioavailable DOM, therefore producing the highest bacterial abundances (Benner *et al.*, 1995), although, experiments with similar high flow conditions did not show the same trends.

5.5 CHAPTER SUMMARY

This study has shown the importance of calibrating ultrafiltration cartridges to verify the nominal molecular weight cut-off of the membrane. The two filter cartridges (1 and 30 kDa) used in this research were expected to have very different MW cut-offs. Instead it was shown that vitamin B-12 with a molecular weight of 1.33 kDa was able to permeate the 1 kDa filter and cytochrome C (NMW of 12 kDa) was retained by the 30 kDa filter. This suggests that the ultrafiltration cartridges had closer MW cut-off than anticipated and it may have been useful to use an intermediate macromolecular standard.

The small size of the nitrate molecule suggests that the concentration should not change during the filtration process. Instead a reduction of nitrate concentration was observed in the 1 kDa permeate, relative to the concentration of the original solution. This suggests that some nitrate is retained by the filter. A similar decrease was not observed from the retentate of the 30 kDa filter. It is thought that either membrane adsorption or electrostatic membrane – ion repulsion may have been stronger in the 1 kDa ultrafiltration cartridge due to the smaller pore size than that of the 30 kDa filter (Mannino and Harvey, 2000; Guo *et al.*, 2001).

The molecular size fractionation of River Test DOM has a variable composition in terms of LMW, HMW and VHMW fractions. None of these fractions was consistently larger than the others. It is possible that the molecular size composition of the river is dependent upon the river flow (Benner *et al.*, 1995), although this was not clearly apparent from these experiments.

The bacterial bioassay experiments showed that there was no statistical difference in bacterial abundance in the three molecular size fractions. This may be dependant on the molecular size composition of the DOM and lack of dominant size fraction. The original concentrations of DOM in the river water in January 2004 were low. If the experiments were performed with a higher DOM concentration then changes in bacterial abundance may have been more apparent.

6 CHAPTER SIX. CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 GENERAL CONCLUSIONS

The overall aim of this research was to determine temporal and spatial changes in dissolved organic matter (DOM) in a high nitrate river/estuarine system and in addition to investigate the bioavailability of riverine DOM relative to its molecular size distribution. Data from an intensive 18 month study of the River Test and estuary was compiled to determine the temporal and spatial variation in DOM (DOC + DON) in relation to dissolved inorganic nitrogen concentrations (nitrate, ammonium) particulate organic nitrogen and carbon in the lower reaches of the River Test and the upper estuary. Few other studies have attempted to assess spatial variations in dissolved organic nitrogen (DON) and carbon (DOC) along a river / estuary continuum and identify (i) the importance of point source inputs along the river and (ii) the influence of intertidal salt marshes on nutrient concentrations. The contribution of DON to riverine nitrogen loads entering UK estuaries remain largely unknown even though it is becoming evident that DON has an important role in estuarine productivity (Seitzinger and Sanders, 1997b). There has been a lack of research into the bioavailability of DON and its potential to contribute to eutrophication.

No other investigations into seasonal variations in DON concentrations have been carried out in UK river and estuarine systems. A clear seasonal trend was not apparent in either the concentration of DON nor the percentage of total nitrogen as DON in the River Test or its estuary. Despite DON being the second largest total nitrogen component contributing up to 7 % in the river and 13 % of the total dissolved nitrogen (TDN) in the Test estuary, there were no similarities in temporal variations when compared with nitrate as the dominant fraction of dissolved nitrogen (Anonymous, 1983; Sanders *et al.*, 1997). The processes affecting DON concentrations on a temporal scale are more complex than those influencing the inorganic nitrogen species. The lack of seasonal variation in DON concentrations in this system was in contrast to that of DOC, which showed a clear temporal variation in both freshwater and saline samples. It appears that DOC and DON in the river and estuary are not correlated and are influenced by different processes such as the preferential breakdown of DON and the removal of DOC in the spring months.

The annual DON load from the River Test entering the estuary was approximately 10 % of the total dissolved nitrogen load. The majority of the dissolved nitrogen load was composed of nitrate, with only 1 % being ammonium. There was no clear relationship between flow and the monthly DON load, although the peak loads (for all dissolved constituents of nitrogen) coincided in December 2002 during high flow rates. This represents the first study of DON load (23 Mmol yr^{-1}) and area-normalised load (1.9 x 10^4 mol km^{-2} yr^{-2}) in a chalk river and was in good agreement with other studies worldwide (14 to 207 Mmol yr-1 DON load; 5.9 to 31.4 mol km^{-2} yr^{-2} area-normalised DON load), although the DON load was fairly low compared to most lowland UK studies (28 to 51 Mmol yr-1 DON load; 1.4 to 3.9 x 10^4 mol km^{-2} yr^{-2} area-normalised load)(Edwards *et al.*, 1996). This study has shown mean nitrate concentrations within the River Test to be 462 µM between 2001 and 2002 which suggests an increase of more than 25 % over the last 25 years (Hydes and Wright, 1999).

This was the first UK study of spatial variations in DON concentrations from a river to estuary system. These spatial variations in DON and DOC along the Test river - estuary continuum indicate a general downstream trend of increasing DON and DOC concentrations. This was in contrast to a clear decrease in nitrate from high concentrations at the source to lower values at the freshwater limit at Redbridge. A downstream increase in ammonium was only apparent during the winter months when rates of biological removal were likely to be at a minimum. The undetectable DON levels at the aquifer fed source of the River Test suggests that ground water and run off are potential diffuse sources, with biological production being an additional internal source and in some months sewage treatment works provided a point source of DON. DOC concentrations at the river source were higher than those measured at the upper limit of the sampling area. This suggests a ground water source of DOC is diluted in the upper catchment and, further downstream, other DOC point source inputs become important as shown by Tipping *et al.* (1997) for the Humber catchment.

Concentrations of DON measured downstream of salt marshes were lower than those determined immediately upstream, suggesting that DON assimilation or ammonification processes were important in this area. DON may also be affected by other physical removal processes including adsorption, flocculation and degradation (Mantoura and Woodward, 1983; Libes, 1992; Mannino and Harvey, 2000; Cauwet, 2002; Moreira-Turcq *et al.*, 2003). Generally, higher concentrations of both DON and DOC were recorded in

freshwater samples relative to saline samples and the nutrient-salinity mixing behaviour was non-conservative in all but a couple of months, indicating DOM was biologically reactive in these areas and that losses and additions occur within the estuary (Goñi *et al.*, 2003). This to be expected in the stratified upper Test estuary, which has longer mixing times than well-mixed estuaries (Cauwet, 2002). Elevated concentrations of DOC were observed downstream compared to upstream of a fish farm on the majority of sampling dates. These increases were probably associated with food wastage, fish excretion and faeces (Gowen and Bradbury, 1987; Wu, 1995; Lin and Yi, 2003), with the largest increases occurring in the late spring and early summer. However, DON concentrations remained unchanged downstream of the fish farm suggesting a decoupling of the processes influencing DON and DOC in this area.

A further aim of this work was to investigate the bioavailability of molecular size fractionated freshwater DON and DOC from the River Test using bacterial bioassays. Initially the calibration of the filters indicated that the difference in nominal molecular weight cut-offs of the filters were closer than expected and compounds 12 kDa were being retained by the 30 kDa filter. There was also the added problem that some nitrate was retained by the 1 kDa filter membrane. This work established that the chemical composition of the river water during winter 2003 – 2004 in the lower River Test varied over time. On the majority of sampling dates DOM was dominated by either low (LMW) or high (HMW) molecular weight material. The very high molecular weight (VHMW) material was a larger fraction of the DON composition compared with <10 % of the DOC of the VHMW material.

An attempt was made to assess the biological availability of molecular size fractions of DOM using a bacterial bioassay approach. Preliminary results from this research showed that bacterial growth was not affected by the various molecular size fractionated DON and DOC. The three bacterial bioassay experiments showed no statistically significant difference between bacterial growth in the 0.2 μm filtered water (total DON and DOC), LMW (< 1 kDa) or LMW plus HMW (< 30 kDa) fractions. This may be dependant on the molecular size composition of the DOM and lack of consistent size fraction composition.

This research has shown that DON is an important fraction of the total nitrogen budget of a UK south coast river contributing up to 10 % of the dissolved nitrogen load. There was no

apparent seasonal variation in DON concentrations within the area surveyed over the 18 months, although in some months sewage treatment works provide a source to the river and the salt marshes in the lower Test remove DON from the river water.

6.2 SUGGESTIONS FOR FURTHER WORK

This study has produced the first comprehensive data set of DON concentrations measured on a monthly basis at a number of positions along the course of a UK river and upper estuarine system. The research has investigated temporal and spatial variations in concentrations but directly linking these variations to specific processes affecting concentrations of DON in the lower river or upper estuary has proved more challenging. The multiple linear regression analysis identified that the variables measured in this study do not contribute to the ability to predict DON, therefore other factors must be of importance. Several point source inputs to the river were investigated in this study but determining the importance of diffuse sources of nutrients is a difficult task. Further investigation into diffuse DON sources such as precipitation, leaf litter decomposition and soil run off sources may identify whether these processes were controlling DON concentrations in the River Test.

In addition, the study has revealed that the salt marshes are an area of importance to nutrient cycling and a potential site of DON removal. An extension of the investigation in this intertidal area would be to monitor DON concentrations on daily timescale, sampling on both high and low tides over a period of several days. These investigations may constrain further the nitrogen load entering the estuary and DON cycling in this area.

Most studies of nutrient inputs to rivers and estuaries have concentrated on measuring dissolved inorganic nitrogen, although this represents an incomplete analysis of the total nitrogen (TN) sources to these systems. UK monitoring agencies measure DIN to ensure compliance with international obligations and legislation but chemical composition or total concentration of DON in rivers and estuaries is mostly unknown. Advances in the analytical determination and characterisation of DON have identified that it is potentially biologically available to phytoplankton and bacteria and it has been linked to the increased occurrence of harmful algal blooms. Since total quantities of nitrogen entering sensitive estuarine waters from UK rivers is currently an underestimate, it is recommended that routine monitoring of DON concentrations is carried out to obtain more accurate estimates

of inputs to estuaries. It is also advised that international agreements are amended to acknowledge the importance of DON to the total nitrogen load entering coastal areas.

Finally it has been shown from this study that temporal variations are observed in the molecular size composition of DON and DOC in the River Test. The current research was limited to an investigating of the composition in winter months. Changes in DOC and DON molecular size composition may be apparent from monthly size fractionation experiments, which could be used to investigate temporal changes in the bioavailability of DOM to bacteria and micro algae in the river and estuarine system.

APPENDICES

Appendix A. Predicted tidal heights in Southampton water on sampling dates July 2001 – December 2002. N.B. all times are given as GMT. Tide heights are relative to chart datum. (UK Hydrographic Office, 2000, 2001)

18/07/2001	**16/01/2002**	**11/07/2002**
01:51 (1.4 m)	00:13 (4.4 m)	04:53 (0.8 m)
08:20 (4.0 m)	06:06 (1.0 m)	11:17 (4.4 m)
14:14 (1.3 m)	12:24 (4.4 m)	17:12 (0.9 m)
20:47 (4.3 m)	18:24 (0.8 m)	23:32 (4.5 m)
21/08/2001	**15/02/2002**	**09/08/2002**
05:58 (0.2 m)	00:24 (4.4 m)	04:40 (0.6 m)
12:17 (4.8 m)	06:20 (0.8 m)	11:02 (4.5 m)
18:20 (0.4 m)	12:35 (4.3 m)	17.01 (0.8 m)
	18:33 (0.7 m)	23:18 (4.6 m)
19/09/2001	**18/03/2002**	**06/09/2002**
05:38 (0.1 m)	00:55 (4.3 m)	03:37 (0.8 m)
11:55 (4.9 m)	06:45 (0.8 m)	10:00 (4.5 m)
18:00 (0.3 m)	13:09 (4.2 m)	15:59 (0.9 m)
	18:56 (0.9 m)	22:16 (4.7 m)
19/10/2001	**15/04/2002**	**08/10/2002**
05:53 (0.3 m)	05:53 (0.6 m)	05:29 (0.1 m)
12:10 (4.8 m)	12:10 (4.3 m)	11:45 (5.0 m)
18:15 (0.4 m)	18:05 (0.7 m)	17:51 (0.3 m)
16/11/2001	**14/05/2002**	**07/11/2002**
04:47 (0.5 m)	05:28 (0.7 m)	05:46 (0.4 m)
11:05 (4.8 m)	11:47 (4.3 m)	12:05 (4.9 m)
17.11 (0.5 m)	17:42 (0.9 m)	18:10 (0.5 m)
23:25 (4.7 m)		
17/12/2001	**14/06/2002**	**06/12/2002**
05:45 (0.9 m)	00:26 (4.4 m)	05:27 (0.7 m)
12:04 (4.5 m)	06:26 (0.8 m)	11:47 (4.7 m)
18:07 (0.8 m)	12:55 (4.4 m)	17:52 (0.6 m)
	18:47 (1.1 m)	

Appendix B. Temperature (^{o}C) measurements at all sampling sites, July 2001 – December 2002. ND indicates no data.

Site	18/07/01	21/08/01	19/09/01	19/10/01	16/11/01	17/12/01	16/01/02	15/02/02	18/03/02
1	14.9	ND	15.7	ND	9.9	8.2	6.0	6.9	10.0
2	14.9	17.1	11.2	13.1	7.3	4.6	6.7	6.6	11.0
3	14.5	16.2	11.8	13.1	7.2	4.7	6.7	6.6	9.9
4	14.6	16.2	11.8	13.1	7.2	4.6	6.0	6.6	12.6
5	14.8	16.2	11.6	13.1	7.2	4.7	6.7	6.6	11.7
6	ND	19.2	15.2	12.2	8.4	6.5	6.7	7.1	10.0
6a	ND	17.2	12.2	11.0	7.5	5.0	6.9	7.1	10.0
7	15.2	17.0	12.3	11.5	7.6	5.0	6.8	7.0	10.0
8	15.4	17.1	12.2	10.9	7.7	5.0	6.8	7.1	10.0
9	14.9	16.4	11.7	12.9	7.4	4.9	6.7	6.7	12.1
10	15.1	16.7	12.1	12.0	7.6	5.1	6.8	7.0	10.0
11	14.6	15.9	11.8	12.6	7.7	5.1	6.9	6.8	12.9
12	15.0	16.0	11.5	12.2	8.3	5.6	7.0	7.0	10.9
13	15.0	16.1	11.7	12.0	8.2	5.4	6.9	7.0	10.6
14	15.2	16.4	11.6	12.4	8.3	5.4	7.2	7.0	10.0
15	14.9	16.0	11.7	12.4	8.4	5.6	7.2	7.0	12.1
16	15.7	19.8	15.7	13.8	9.0	5.6	6.8	7.5	10.0
17	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	ND	ND	ND	ND	ND	ND	ND	ND	ND

Site	15/04/02	14/05/02	14/06/02	11/07/02	09/08/02	06/09/02	08/10/02	07/11/02	06/12/02
1	10.7	13.4	5.8	ND	18.9	19.6	16.4	12.5	9.1
2	10.0	12.4	14.9	16.0	16.8	17.6	12.6	10.3	8.2
3	9.8	12.3	14.8	15.9	16.8	16.2	12.6	10.4	8.1
4	9.9	12.2	14.8	ND	16.8	16.3	12.6	10.3	8.2
5	9.8	12.2	14.8	ND	16.7	16.2	12.6	10.3	8.2
6	11.2	13.2	15.4	ND	18.7	17.7	14.5	12.1	8.8
6a	11.0	12.9	15.3	ND	16.8	16.6	12.7	10.5	8.5
7	10.8	12.4	15.2	ND	18.6	16.6	12.6	10.4	8.5
8	10.9	12.5	15.3	ND	16.6	16.5	12.6	10.4	8.5
9	10.2	12.2	14.8	ND	16.6	16.3	12.4	10.3	8.3
10	10.6	12.2	14.9	ND	16.4	16.3	12.5	10.3	8.5
11	10.2	12.2	14.5	ND	16.1	16.0	12.4	9.9	8.5
12	10.6	12.2	14.6	ND	15.5	15.5	12.2	10.1	8.7
13	10.3	12.2	14.5	ND	15.4	15.6	12.2	10.1	8.7
14	10.6	12.2	14.8	ND	15.4	15.7	12.3	10.2	8.7
15	10.2	12.2	14.5	ND	15.5	15.4	12.2	10.1	8.8
16	11.6	13.4	6.0	ND	18.0	17.7	16.4	11.5	9.0
17	ND	ND	8.1	17.5	18.2	19.3	15.7	12.5	10.3
18	ND	ND	ND	ND	19.6	19.7	16.3	13.0	10.7

Appendix C. Conductivity (µS cm^{-1}) measurements at all sampling sites, July 2001 – December 2002. ND indicates no data.

Site	18/07/01	21/08/01	19/09/01	19/10/01	16/11/01	17/12/01	16/01/02	15/02/02	18/03/02
1	3850	ND	40100	ND	22900	31200	31500	3300	2560
2	3850	3960	757	1054	443	345	432	378	400
3	435	548	419	516	373	347	354	346	379
4	437	513	403	497	370	345	361	305	360
5	439	548	404	497	370	347	361	354	358
6	ND	34000	35300	1506	10690	193	9680	796	375
6a	ND	5830	443	566	384	353	379	796	370
7	458	555	429	522	385	357	376	378	379
8	458	555	427	521	379	356	376	379	380
9	454	556	408	523	382	357	377	375	383
10	457	561	432	523	387	359	377	377	372
11	453	565	422	523	388	362	378	378	380
12	460	566	414	519	394	365	380	379	396
13	459	565	426	522	382	362	379	380	383
14	460	567	414	522	395	363	381	387	396
15	460	566	430	523	396	366	381	387	390
16	551	38700	40000	16060	13800	9140	5300	7430	2640
17	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	ND	ND	ND	ND	ND	ND	ND	ND	ND

Site	15/04/02	14/05/02	14/06/02	11/07/02	09/08/02	06/09/02	08/10/02	07/11/02	06/12/02
1	34900	35400	36100	36300	36700	44300	48000	39000	17300
2	447	426	356	648	608	16700	1285	720	599
3	400	370	365	533	460	564	584	403	570
4	399	370	432	532	460	565	551	439	568
5	399	370	435	534	459	564	567	460	570
6	7850	2180	460	2400	27200	17220	27700	32100	15530
6a	413	403	424	553	560	944	620	770	588
7	411	406	456	572	456	561	580	565	592
8	412	409	458	567	458	562	581	571	592
9	406	407	450	564	457	564	580	510	590
10	411	366	465	569	461	566	583	565	547
11	406	412	456	570	517	566	584	580	596
12	409	410	466	578	457	572	587	568	601
13	408	414	466	576	456	571	586	592	600
14	412	414	462	574	460	570	584	579	602
15	409	413	456	561	456	572	587	575	602
16	17640	6730	6120	12570	20600	14060	48000	21300	13480
17	ND	ND	3670	13310	14310	36600	42900	39000	41800
18	ND	ND	ND	ND	43300	44500	38100	47900	47900

Appendix D. Salinity measurements at all sampling sites, July 2001 – December 2002. ND indicates no data.

Site	18/07/01	21/08/01	19/09/01	19/10/01	16/11/01	17/12/01	16/01/02	15/02/02	18/03/02
1	1.7	1.6	31.0	0.0	21.9	28.3	20.2	16.0	15.7
2	1.7	0.0	0.3	0.0	0.1	0.0	0.1	0.2	0.3
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	ND	0.7	0.0	0.4	0.0	0.1	0.0	0.0
6	ND	0.8	26.8	0.7	9.7	0.0	3.9	0.7	0.0
6a	ND	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
7	0.0	ND	ND	0.0	0.0	0.0	0.0	0.0	0.0
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ND
9	0.0	ND	0.0	0.1	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11	0.0	ND	ND	0.0	0.0	0.0	0.0	0.0	0.0
12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13	0.0	ND	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14	0.0	ND	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	0.0	24.2	31.0	10.4	16.0	6.8	3.1	5.5	2.2
17	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	ND	ND	ND	ND	ND	ND	ND	ND	ND

Site	15/04/02	14/05/02	14/06/02	11/07/02	09/08/02	06/09/02	08/10/02	07/11/02	06/12/02
1	30.5	24.6	27.0	22.3	19.7	17.8	30.2	19.2	10.6
2	0.1	0.0	0.0	0.1	0.1	3.0	0.4	0.1	0.0
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	3.7	0.5	0.0	1.1	9.5	10.0	16.5	17.9	4.4
6a	0.0	0.0	0.0	0.0	0.5	0.1	0.0	0.1	0.0
7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	13.2	4.2	3.9	7.1	11.2	11.1	28.0	14.2	6.8
17	ND	ND	0.0	7.6	13.0	21.7	26.4	25.5	12.0
18	ND	ND	ND	ND	27.9	31.4	31.6	32.4	30.4

Appendix E. Shimadzu 5000A and Antek 705E instrumental conditions

Blank water	UV cleaned (4 hr) Milli-Q water
Acidification of samples	50 µl of 10 % HCl made in blank water
Cleaning of sample vials	10 % H_2O_2 and combusted (4 hr at 550 ^oC)
Shimadzu 5000A settings (TOC)	
Carrier gas	Oxygen (ultra pure 99.999 %)
Gas flow rate through TOC 5000A	150 ml min^{-1}
TC catalyst	Normal sensitivity
Detector	Infra red gas analyser detects CO_2
Furnace temperature	680 ^oC
Syringe volume	250 µl
Injection volume	100 µl
No. washes	4
No. injections	4
Injection interval	240 seconds
TOC range	1
Antek 705 E settings (TDN)	
Furnace temperature	900 ^oC
TDN sensitivity	10
Detector	Chemiluminescent detection of NO_2

Appendix F. Sequence of analysis of standards, blanks and samples for HTCO determination of DOC and TDN. Nitrogen concentrations are given for the caffeine standards, there is a 1:2 ratio of N:C in caffeine therefore carbon concentrations are twice that of nitrogen.

Blank (UV irradiated Milli-Q water)

Blank

500 µM caffeine (mid range standard to check for drift throughout batch)

700 µM caffeine (high standard)

200 µM caffeine (medium-low standard)

100 µM caffeine (low standard)

Blank

500 µM caffeine

500 µM potassium nitrate (identical concentration to previous caffeine standard to check oxidation efficiency)

A. Set of samples

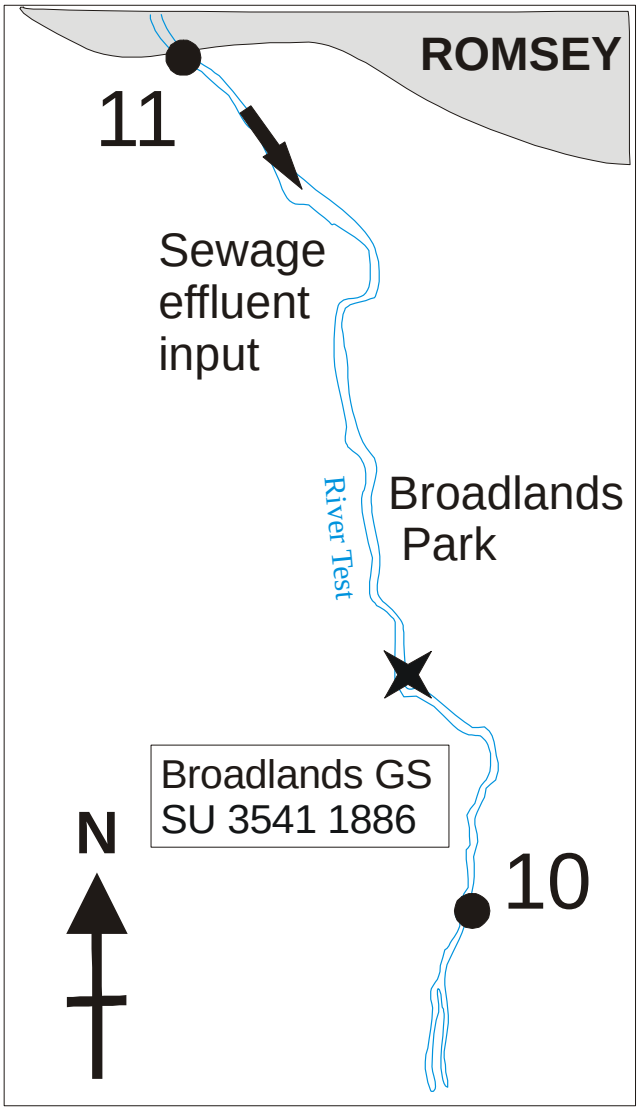
B. Blank

C. 500 µM caffeine

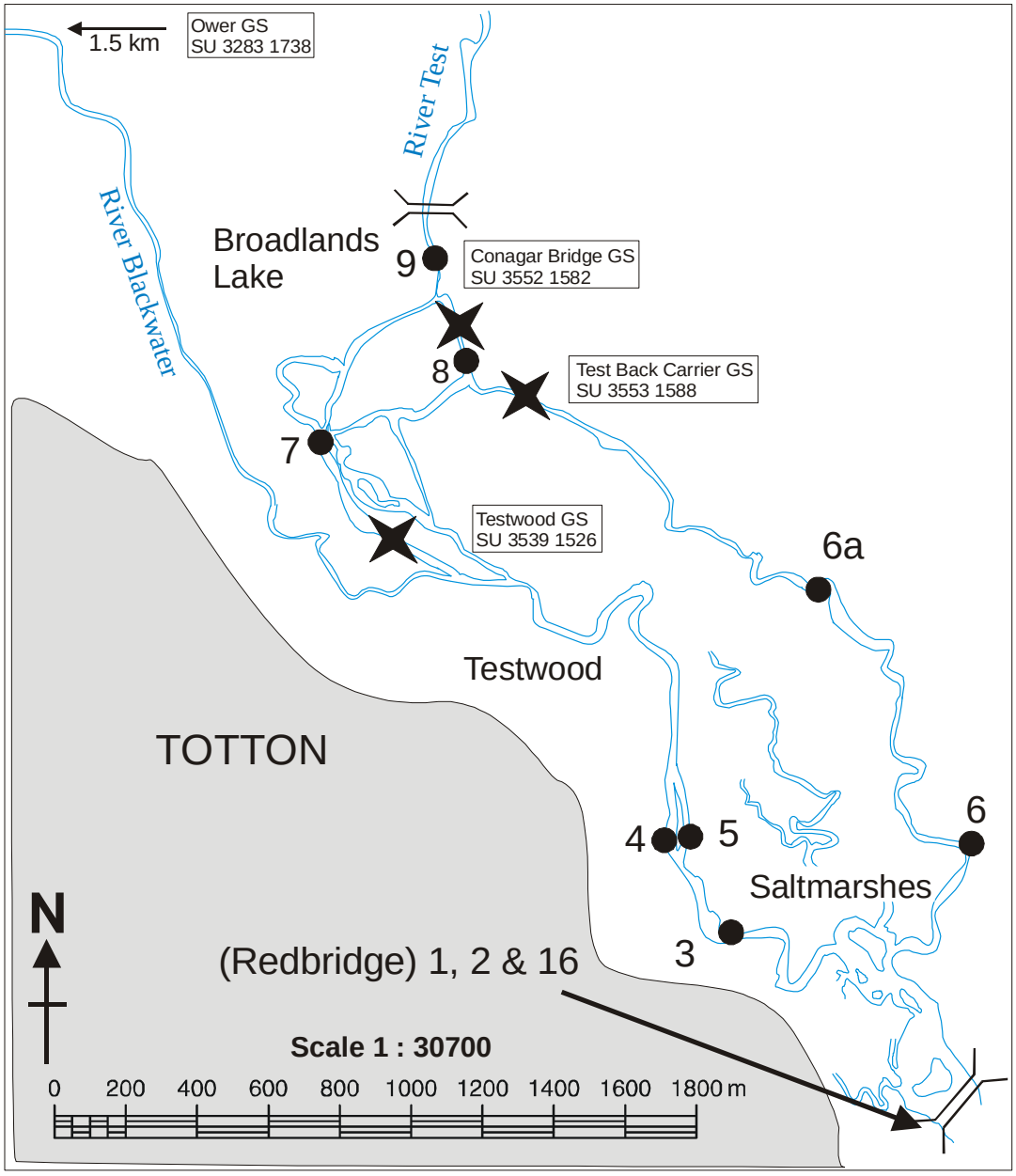
D. 500 µM potassium nitrate (identical concentration to previous caffeine standard to check oxidation efficiency)

Repeat A, B, C and D as many times as necessary

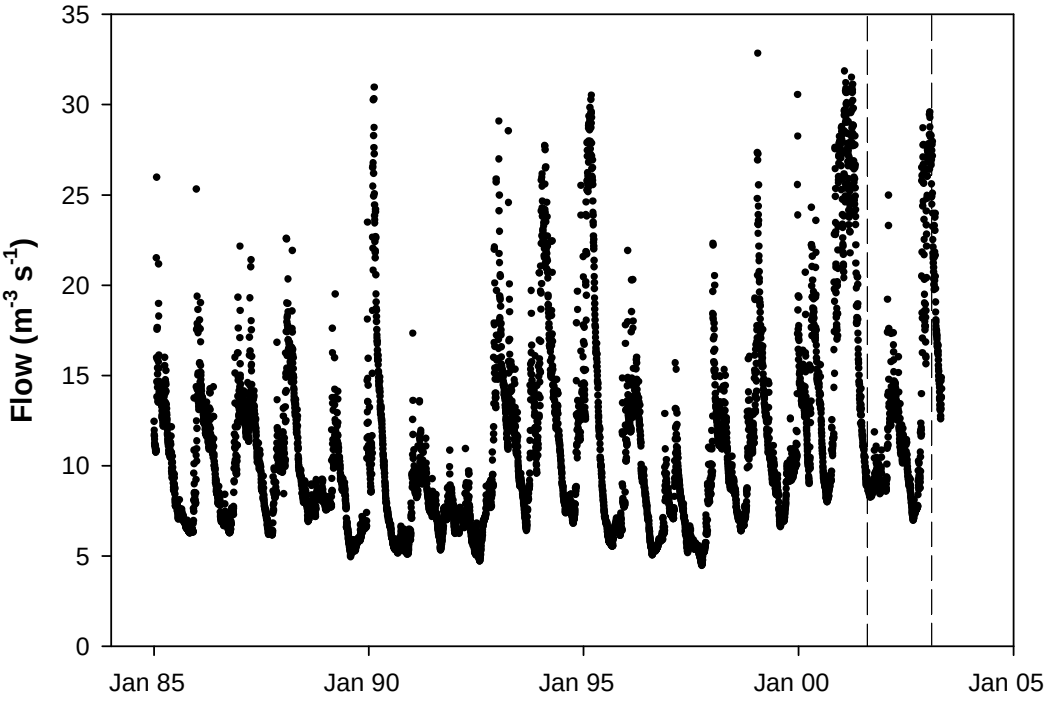
Appendix G. Map of Romsey to Longbridge. Location of Broadlands flow gauging station (SU 3541 1886) and input from Greenhill STW relative to nutrient sampling sites 10 and 11. Filled dots indicate sampling sites and filled crosses are Environment Agency flow gauging stations.



Appendix H. Map of lower Test (Broadlands Lake to Redbridge); channel pattern and location of gauging stations. Filled dots indicate sampling sites and filled crosses are Environment Agency flow gauging stations.



Appendix I. Daily mean river flow at Broadlands (SU 3541 1886) on the River Test from January 1985 to May 2003. The section between the dotted lines indicates the duration of the 18-month sampling programme from July 2001 to December 2002.



Appendix J. Environment Agency nutrients measured at Longbridge (SU 3550 1780) during the Harmonised Monitoring Scheme (HMS).

Date	Nitrate	Ammonium
	(μM)	(μM)
19/06/2001	499	4.4
06/08/2001	454	5.0
30/08/2001	464	5.1
02/10/2001	432	4.1
06/11/2001	480	4.3
29/11/2001	492	6.0
10/12/2001	527	5.6
17/12/2001	526	7.1
21/01/2002	494	7.7
25/02/2002	514	5.1
19/03/2002	471	5.1
16/04/2002	511	2.6
31/05/2002	469	4.6
04/07/2002	475	4.9
31/07/2002	448	2.5
29/08/2002	462	6.2
02/10/2002	445	4.9
29/10/2002	453	3.8
23/11/2002	426	6.8
18/12/2002	536	5.4

Appendix K. Research outputs

Conference oral presentations:

- Liverpool workshop on dissolved organic matter, "Temporal and spatial changes in DOC and DON in a river / estuarine system", Liverpool, July 2004
- American Society of Limnology and Oceanography (ASLO) / The Oceanography Society (TOS) at the Ocean Research Conference, "Chemical composition and bioavailability of DOM in a river / estuarine system", USA, February 2004
- GANE thematic programme annual meeting, "Behaviour of DON in the lower reaches of a chalk-bed river", Edinburgh, September 2003
- Postgraduate Research in Marine Sciences (PRMES), "Dissolved organic nitrogen in the Upper Test estuary and River Test", Southampton, April 2003
- GANE thematic programme annual meeting, "Dissolved inorganic and organic nitrogen; spatial and temporal distribution in an aquifer fed chalk river", Nottingham, September 2002

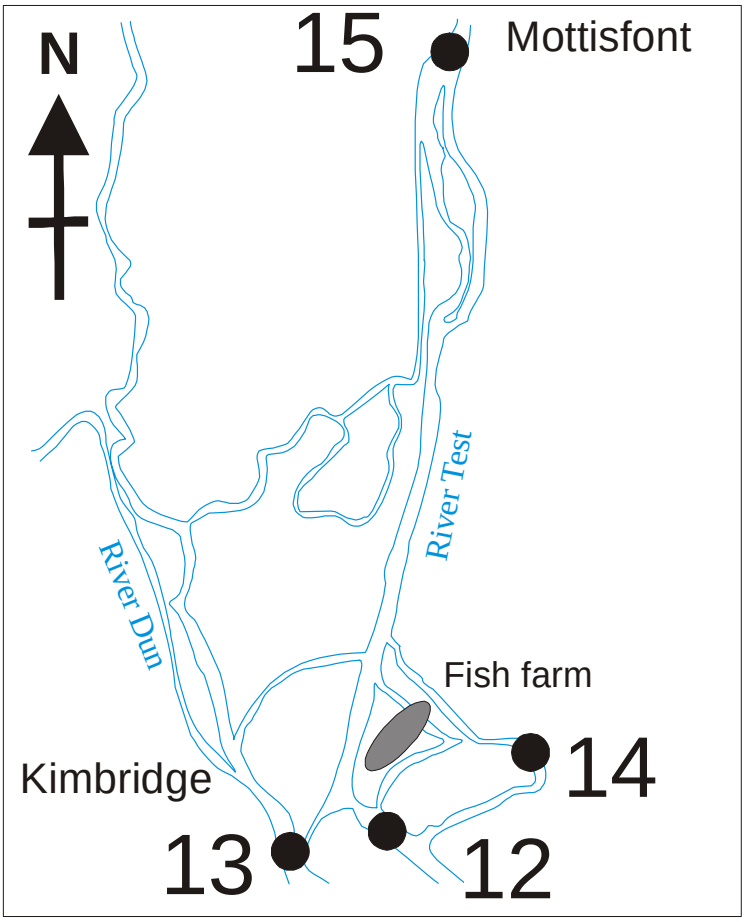
Conference poster presentations:

- GANE – too much of a good thing? Programme finale, "Dissolved organic nitrogen in the Test river and estuary", London, February 2004 (co-author)
- Challenger Society UK Marine Science Centenary Conference, "Dissolved inorganic and organic nitrogen, spatial and temporal distributions in an aquifer fed chalk river", Plymouth, September 2002
- First GANE thematic programme annual meeting, "Spatial distribution of ammonium, nitrate and dissolved organic nitrogen in the River Test, Hampshire, UK", Bangor, September 2001

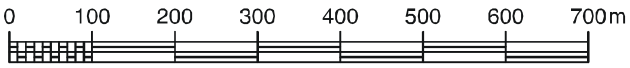
Publications:

Homewood, J. M., Purdie, D. A and Shaw, P. J., 2004, Influence of sewage inputs and fish farm effluents on dissolved nitrogen species in a chalk river, *Water, Air and Soil Pollution; Focus* 4, 117-125

Appendix L. Map of Kimbridge fish farm relative to sites 12 and 15



Scale 1:12271



Appendix M. River Test at (a) Broadlands Lake (site 9) and Redbridge (sites 1, 2 and 16) during (b) low and (c) high tide on 24th July 2001.

(A)

(B)

(C)

Appendix N. Procedure for calibration of ultrafiltration cartridges

- Assemble ultrafiltration cartridge in cartridge holder and flush out 0.1 N NaOH cleaning solution using fresh Milli Q water. Initially flush 2 L through the retentate line, followed by 6 L through the permeate line and the final 1 L through the retentate line.
- Check the pH of the permeate line is > pH 5 and proceed with step 3. If not > pH 5, return to flushing procedure step 1.
- Give the system a final flush with 1 L UV cleaned water, passing 200 ml through the retentate line, 600 ml through the permeate and the final 200 ml through the retentate line.
- Weigh out calibration solution to produce a volume of 5 L.
- Sub-sample from the calibration solution to determine the initial concentration
- Set aside 4 L of the calibration solution in a separate flask
- Use the remaining calibration solution to initially flush the ultrafiltration system (using similar flush ratios to step 1.). Then drain permeate and retentate lines as well as ultrafiltration cartridge.
- Place the feed tube into the container of 4 L calibration solution.
- At a flow rate of 1 L/min turn the retentate screw to achieve a pressure of 1.3 psi.
- Collect duplicate samples from the permeate and retentate flasks at the following concentration factors 2, 4, 10 and 20 and store frozen for later TDN and DOC determination.
- Flush the cartridge as in step 1 and pump through 0.1 N NaOH solution at the highest flow rate before sealing ends and storing in the fridge.

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