

# 1 A multi-stable isotope framework to understand eutrophication in aquatic ecosystems

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## 10 Abstract

11 Eutrophication is a globally significant challenge facing aquatic ecosystems, associated with human  
12 induced enrichment of these ecosystems with nitrogen (N) and phosphorus (P). However, the limited  
13 availability of inherent labels for P and N has constrained understanding of the triggers for  
14 eutrophication in natural ecosystems and appropriate targeting of management responses. This paper  
15 proposes and evaluates a new multi-stable isotope framework that offers inherent labels to track  
16 biogeochemical reactions governing both P and N in natural ecosystems. The framework couples  
17 highly novel analysis of the oxygen isotope composition of phosphate ( $\delta^{18}\text{O}_{\text{PO}_4}$ ) with dual isotope  
18 analysis of oxygen and N within nitrate ( $\delta^{15}\text{N}_{\text{NO}_3}$ ,  $\delta^{18}\text{O}_{\text{NO}_3}$ ) and with stable N isotope analysis in  
19 ammonium ( $\delta^{15}\text{N}_{\text{NH}_4}$ ). The River Beult in England is used as an exemplar system for initial evaluation  
20 of this framework. Our data demonstrate the potential to use stable isotope labels to track the input  
21 and downstream fate of nutrients from point sources, on the basis of isotopic differentiation for both P  
22 and N between river water and waste water treatment work effluent (mean difference = +1.7‰ for  
23  $\delta^{18}\text{O}_{\text{PO}_4}$ ; +15.5‰ for  $\delta^{15}\text{N}_{\text{NH}_4}$  (under high flow); +7.3‰ for  $\delta^{18}\text{O}_{\text{NO}_3}$  and +4.4‰ for  $\delta^{15}\text{N}_{\text{NO}_3}$ ). Stable  
24 isotope data reveal nutrient inputs to the river upstream of the waste water treatment works that are  
25 consistent with partially denitrified sewage or livestock sources of nitrate ( $\delta^{15}\text{N}_{\text{NO}_3}$  range = +11.5 to  
26 +13.1‰) and with agricultural sources of phosphate ( $\delta^{18}\text{O}_{\text{PO}_4}$  range = +16.6 to +19.0‰). The

27 importance of abiotic and metabolic processes for the in-river fate of N and P are also explored  
28 through the stable isotope framework. Microbial uptake of ammonium to meet metabolic demand for  
29 N is suggested by substantial enrichment of  $\delta^{15}\text{N}_{\text{NH}_4}$  (by 10.2‰ over a 100m reach) under summer  
30 low flow conditions. Whilst the concentration of both nitrate and phosphate decreased substantially  
31 along the same reach, the stable isotope composition of these ions did not vary significantly,  
32 indicating that concentration changes are likely driven by abiotic processes of dilution or sorption.  
33 The in-river stable isotope composition and the concentration of P and N were also largely constant  
34 downstream of the waste water treatment works, indicating that effluent-derived nutrients were not  
35 strongly coupled to metabolism along this in-river transect. Combined with in-situ and laboratory  
36 hydrochemical data, we believe that a multi-stable isotope framework presents a powerful approach  
37 for understanding and managing eutrophication in natural aquatic ecosystems.

38

## 39 **1. Introduction**

40 Perhaps the most significant challenge facing aquatic ecosystems globally is cultural eutrophication  
41 (Schindler, 2012), the process of ecosystem change triggered by human induced enrichment of  
42 ecosystems with phosphorus (P) and nitrogen (N). Given the adverse ecological, social and economic  
43 impacts associated with eutrophication (Dodds et al., 2009; Pretty et al., 2003), significant research  
44 efforts have been directed towards understanding the causes of this process and targeting mitigation  
45 strategies. In the context of aquatic ecosystems, two long-standing paradigms suggest that primary  
46 production is limited by an individual nutrient element which thereby represents the trigger for  
47 eutrophication. In freshwaters the focus has been on limitation by the availability of P (e.g. Likens,  
48 1972; Schindler, 1977), whilst within estuarine and coastal marine ecosystems the focus has been on  
49 N limitation (e.g. Ryther and Dunstan, 1971; Howarth, 1988). However, these paradigms have been  
50 subject to growing debate, stimulated by evidence of N limitation in freshwaters (e.g. Mischler et al.,  
51 2014; James et al., 2003), N/P co-limitation in freshwaters (e.g. Xu et al., 2010; Conley et al., 2009),  
52 or P limitation in marine/estuarine waters (e.g. Blomqvist et al, 2004).

53 This debate reflects uncertainty regarding a number of the fundamental questions that surround  
54 eutrophication and appropriate responses to eutrophication (Smith and Schindler, 2009). An important  
55 source of this uncertainty is reliance on bioassays and mesocosms as the experimental basis for  
56 understanding nutrient limitation and eutrophication in aquatic ecosystems. These experimental  
57 approaches may not accurately reflect the large-scale, long-term processes that govern eutrophication  
58 in natural ecosystems, resulting in a bias towards identification of proximate rather than ultimate  
59 limiting nutrients (Vitousek et al., 2010) and data that do not scale successfully to natural ecosystems  
60 (Schindler, 2012). Past reliance on bioassays and mesocosms partly reflects the lack of inherent  
61 tracers that can be used to understand the sources and the reaction pathways which control P and N  
62 biogeochemistry in natural ecosystems (Karl, 2000). In this paper, we propose and evaluate a new  
63 multi-stable isotope framework that offers inherent tracers for N and P within aquatic ecosystems.  
64 Whilst multi-stable isotope approaches are increasingly used in other research fields, for example  
65 employing the stable isotopes of nitrate and sulphate in combination (e.g. Mayer, 2005; Kaown et al.,  
66 2009; Urresti-Estalaa et al., 2015) or combining stable isotope analyses in boron and nitrate (e.g.  
67 Briand et al., 2013), similar frameworks are yet to be developed in the context of P and N  
68 biogeochemistry within aquatic ecosystems.

69 Dual-isotope approaches for nitrate ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ) and stable ammonium isotope analyses  
70 ( $\delta^{15}\text{N}_{\text{NH}_4}$ ) have been used to understand sources and reaction mechanisms for these ions in both  
71 groundwater and surface water (e.g. Böttcher et al., 1990; Böhlke and Denver 1995; Wassenaar 1995;  
72 Kendall 1998; Silva et al., 2000; Heaton et al., 2012; Goddy et al., 2014). Biogeochemical cycling of  
73 P in aquatic ecosystems has previously been examined using the radioactive isotopes  $^{32}\text{P}$  and  $^{33}\text{P}$  (e.g.  
74 Benitez-Nelson, 2000; Benitez-Nelson and Karl, 2002). However, the use of radioisotopes is  
75 constrained by short isotope half-lives, perturbation of experimental systems associated with labelling,  
76 or the use of incubations which omit irregular events in natural ecosystems, such as seasonal algal  
77 blooms (Levine et al., 1986; Thingstad et al., 1993; Benitez-Nelson, 2000). Stable isotope analyses  
78 cannot be conducted on the P atom in P-containing compounds, because  $^{31}\text{P}$  is the only stable P  
79 isotope. However, because P is often bound strongly to oxygen (O) in the dissolved inorganic

80 phosphate ion (Blake et al., 1997), hereafter  $P_i$ , attention has recently focussed on whether the stable  
81 isotope composition of O in  $P_i$  ( $\delta^{18}O_{PO_4}$ ) can provide new insights into sources and biogeochemical  
82 cycling of P in the environment (e.g. Young et al., 2009; Tamburini et al., 2014; Gooddy et al., 2015).  
83 The basis to the use of  $\delta^{18}O_{PO_4}$  in aquatic ecosystems has recently been reviewed by Davies et al.  
84 (2014). Briefly, because the P-O bonds in  $P_i$  are resistant to inorganic hydrolysis under typical  
85 temperature and pressure conditions in the Earth's surface water and groundwater ecosystems (O'Neil  
86 et al., 2003), negligible O isotope exchange occurs between  $P_i$  and water within these ecosystems  
87 without biological mediation (Tudge, 1960; Blake et al., 1997). Under such abiotic conditions,  $\delta^{18}O_{PO_4}$   
88 may therefore reflect the isotope composition of P sources to an ecosystem. In contrast, enzyme-  
89 catalysed reactions cleave P-O bonds leading to fractionation between the isotopes of O in  $P_i$  and O in  
90 a surrounding fluid, either within a cell or within the extracellular environment (Blake et al., 2005).  
91 Intracellular metabolism of P involving the inorganic pyrophosphatase enzyme results in rapid,  
92 temperature-dependent equilibrium fractionation between O in  $P_i$  and O within the intracellular fluid,  
93 the latter is expected to be identical in O-isotope composition to water-O in the extracellular  
94 environment. Given sufficient intracellular-extracellular exchange of P to maintain non-lethal  
95 intracellular P concentrations, a temperature-dependent equilibrium will be established between  
96  $\delta^{18}O_{PO_4}$  and water-O in the extracellular environment. The equilibrium oxygen isotope fractionation  
97 between dissolved inorganic phosphate and water ( $\alpha_{PO_4-H_2O}$ ) at surface temperatures has recently been  
98 determined (Chang and Blake, 2015), using laboratory solutions catalyzed by the inorganic  
99 pyrophosphatase enzyme. These authors derived the equation:

100

$$101 \quad 10^3 \ln \alpha_{PO_4-H_2O} = 14.43 \times (10^3/T) - 26.54 \quad (1)$$

102

103 where T is in degrees Kelvin. Since:

104

$$105 \quad \alpha_{PO_4-H_2O} = (\delta^{18}O_{PO_4} + 1000) / (\delta^{18}O_{H_2O} + 1000) \quad (2)$$

106

107 by combining 1 and 2 above, expected equilibrium  $\delta^{18}\text{O}_{\text{PO}_4}$  values may be calculated from:

108

$$109 \quad \delta^{18}\text{O}_{\text{PO}_4} = (\delta^{18}\text{O}_{\text{H}_2\text{O}} + 1000) \times e^{[14.43 \times (10^3/T) - 26.54]/1000} - 1000 \quad (3)$$

110

111 However, only limited research has explored the use of  $\delta^{18}\text{O}_{\text{PO}_4}$  in aquatic ecosystems, particularly  
112 within freshwater ecosystems. We are not aware of any research to date that has evaluated whether a  
113 multi-stable isotope approach has the potential to provide new insights into the controls on P and N  
114 biogeochemistry within natural ecosystems. Therefore, the objectives of our research were to: i)  
115 develop and apply a multi-stable isotope approach for N and P in freshwater ecosystems; and ii)  
116 evaluate the insights into the sources and reaction mechanisms controlling P and N biogeochemistry  
117 in freshwater ecosystems that can be provided through a multi-stable isotope approach.

118

## 119 **2. Materials and methods**

### 120 **2.1 Study area**

121 The River Beult which rises near Ashford in Kent, UK was used as an exemplar system to evaluate  
122 the multi-stable isotope framework. The Beult is the largest tributary of the River Medway and the  
123 only riverine Site of Special Scientific Interest (SSSI) in the county. Landuse within the Beult  
124 catchment is predominantly rural, with scattered settlements and an urban land coverage of <1% of  
125 the total catchment area. The catchment is predominantly underlain by a thick clay formation (Weald  
126 Clay), largely excluding exchange between groundwater and river water. However, there is evidence  
127 that some groundwater discharge to surface waters may occur in the catchment, either where small  
128 areas of limestone outcrop or where the Weald Clay is discontinuous (Lapworth et al., 2009).  
129 Elevated concentrations of P are found widely within the catchment. For example in a survey  
130 conducted in 2008, 75% of surface waters were found to exceed 100  $\mu\text{g PO}_4\text{-P/L}$  (Lapworth et al.,  
131 2013). Elevated P concentrations place the SSSI in an “unfavourable condition” and exceed target  
132 water quality standards under the European Water Framework Directive (WFD, 2000). Elevated

133 nitrate ( $\text{NO}_3$ ) concentrations are also of concern, with many sites exceeding 30 mg  $\text{NO}_3/\text{L}$  and  
134 therefore exceeding the surface water drinking directive limit of 25 mg  $\text{NO}_3/\text{L}$  (Council Directive  
135 75/440/EEC) and the mean annual concentration for the European Environment Agency's river basin  
136 district (RBD) classification (Class 5 for the study RBD). Given the predominant landuse within the  
137 catchment, agricultural sources coupled with effluent from rural waste water treatment works  
138 (WwTWs) are hypothesised to dominate N and P loads delivered to surface waters in the Beult  
139 catchment (Lapworth et al. 2013). However, the roles of these nutrient sources in controlling  
140 productivity and eutrophication risk in the catchment remain uncertain, as they do within many  
141 aquatic ecosystems globally.

## 142 **2.2 Sites and sampling**

143 A c.200m reach along the River Beult to the south east of the town of Sutton Valence was sampled  
144 during this research (Fig. 1). A total of seven sampling sites (SV1-SV7) were established along an in-  
145 river transect that ran both upstream and downstream of Sutton Valence WwTW (Table 1). Samples  
146 were collected from these sites twice in a six month period, to provide a seasonal contrast between  
147 low flow (September 2013) and high flow (January 2014) conditions. River water samples were  
148 collected from the centre of the flowing water course at each site using a submersible pump, ensuring  
149 that the inlet of the pump did not disturb river bed sediments during sampling. On-site parameters  
150 (dissolved oxygen (DO), pH, temperature and specific electrical conductance (SEC)) were measured  
151 and, where appropriate, were allowed to stabilise prior to sampling. DO, SEC and pH were measured  
152 in a flow-through cell to obtain representative field values. Samples for analysis of chloride, N  
153 species, soluble reactive P (SRP) and total dissolved P (TDP) were 0.45  $\mu\text{m}$  filtered in the field and  
154 collected in 30 mL plastic bottles. Samples for total P (TP) were not filtered and also collected in 30  
155 mL plastic bottles. All samples for isotope analysis were also filtered in the field at 0.45  $\mu\text{m}$  using  
156 high capacity filters. Samples for  $\delta^{15}\text{N}_{\text{NO}_3}$ ,  $\delta^{15}\text{N}_{\text{NH}_4}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  determination were filtered into 1 L  
157 plastic bottles; the samples for  $\delta^{15}\text{N}_{\text{NH}_4}$  determination were acidified in the field with concentrated  
158 HCl to pH 2-4. Samples for  $\delta^{18}\text{O}_{\text{PO}_4}$  determination were filtered into 10 L plastic bottles. Samples for

159 water-oxygen isotope analysis ( $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ ) were collected in 10 mL glass bottles with rubber sealing  
160 caps.

161 *[Fig. 1. Location of the Beult catchment in England, UK (a) and schematic map of sample*  
162 *locations along a section of the river (b)]*

### 163 **2.3 Hydrochemical analyses**

164 Soluble reactive P concentration, a measure of the inorganic monomeric and easily-hydrolysable P in  
165 a sample, was determined colorimetrically using the method of Murphy and Riley (1962) as modified  
166 by Neal et al. (2000). Total phosphorus concentration, the combination of TDP and particulate P  
167 concentrations, was determined by the method of Eisenreich et al. (1975) on unfiltered samples,  
168 whilst TDP concentration was determined using the same method but on filtered aliquots. Samples  
169 were analysed for the concentrations of Cl,  $\text{NO}_3$  and nitrite ( $\text{NO}_2$ ) using ion chromatography (IC), and  
170 for ammonium ( $\text{NH}_4$ ) concentration by flow colorimetry.

### 171 **2.4 Sample preparation for isotope analysis**

172 Nitrate was separated from the sample matrix using anion exchange resins and prepared as silver  
173 nitrate using a method based on Chang et al. (1999). Ammonium was converted to ammonium  
174 sulphate on acidified quartz filter papers using a static ammonia diffusion technique (adapted from  
175 Sigman et al., 1997).

176 We developed and applied a new method to isolate  $\text{P}_i$  from water samples and precipitate silver  
177 phosphate ( $\text{Ag}_3\text{PO}_4$ ) for isotope analysis, shown in Fig. 2 and described in detail in Lapworth et al.  
178 (2014). Samples were processed within 24 h of collection and were stored in the dark at 4 °C prior to  
179 processing. In brief, the majority of dissolved organic matter in a sample is first removed using an  
180 organic exchange resin and  $\text{P}_i$  was then isolated from the remaining matrix using an anion exchange  
181 resin. Phosphate was eluted from the anion exchange resin and chromatographically separated from  
182 competing anions using 0.3 M KCl. Eluted fractions containing phosphate are then processed using a  
183 modified McLaughlin et al. (2004) method to produce a final  $\text{Ag}_3\text{PO}_4$  precipitate for  $\delta^{18}\text{O}_{\text{PO}_4}$  analysis.

184 Any residual organic matter remaining on the  $\text{Ag}_3\text{PO}_4$  precipitate is removed by treatment with 15%  
185 hydrogen peroxide. We believe that the method shown in Fig.2 represents an advance over alternative  
186 sample preparation protocols (e.g. repeated  $\text{CePO}_4$  precipitation, Li et al. (2011)), in that it  
187 successfully prevents contamination of the final  $\text{Ag}_3\text{PO}_4$  precipitate with organic compounds (see  
188 section 2.5 below) whilst also maintaining the final  $\text{Ag}_3\text{PO}_4$  yield. The method reported in Fig. 2 is a  
189 multi-stage process (c. 14 days in total) and was carried out in batches of eight samples.

190 *[Fig. 2. Schematic of the modified McLaughlin et al. (2004) protocol used to process water samples*  
191 *for  $\delta^{18}\text{O}_{\text{PO}_4}$  analysis].*

## 192 **2.5 Mass spectrometry**

193 The ratio  $^{15}\text{N}/^{14}\text{N}$  in  $\text{NH}_4$  and  $\text{NO}_3$  was analysed by combustion in a Flash 1112 EA on-line to a Delta  
194 Plus XL mass spectrometer (ThermoFinnigan, Bremen, Germany), with  $\delta^{15}\text{N}$  values versus  
195 atmospheric  $\text{N}_2$  calculated by comparison with standards IAEA N-1 and N-2 assuming these had  $\delta^{15}\text{N}$   
196 values of +0.4‰ and +20.3‰, respectively. Analytical precision (1 SD) was typically <0.8‰, from  
197 repeat analysis of a sample.  $^{18}\text{O}/^{16}\text{O}$  ratios of  $\text{NO}_3$  were analysed by thermal conversion to CO gas at  
198 1400 °C in a TC-EA on-line to a Delta Plus XL mass spectrometer (ThermoFinnigan, Bremen,  
199 Germany), with  $\delta^{18}\text{O}$  values versus VSMOW calculated by comparison with standard IAEA- $\text{NO}_3$   
200 assuming it had a  $\delta^{18}\text{O}$  value of +25.6‰. Analytical precision (1 SD) was typically <1.2‰.

201  $^{18}\text{O}/^{16}\text{O}$  ratios of  $\text{Ag}_3\text{PO}_4$  were analysed by thermal conversion to CO gas at 1400 °C in a TC-EA on-  
202 line to a Delta Plus XL mass spectrometer (ThermoFinnigan, Bremen, Germany). The  $\delta^{18}\text{O}_{\text{PO}_4}$  value  
203 versus VSMOW was calculated by comparison with an internally run laboratory standard (Alfa Aesar  
204 silver phosphate, 99%). In the absence of an international  $\text{Ag}_3\text{PO}_4$  reference material, we derived the  
205  $\delta^{18}\text{O}$  value of the laboratory standard by comparison with the  $\text{Ag}_3\text{PO}_4$  standard ‘B2207’ (supplied by  
206 Elemental Microanalysis Ltd, Okehampton, England), which has a certified  $\delta^{18}\text{O}$  value of +21.7‰  
207 versus VSMOW. Any organic contamination of the  $\text{Ag}_3\text{PO}_4$  produced using the protocol in Fig 2. was  
208 deemed to be negligible, based on CO yields of the  $\text{Ag}_3\text{PO}_4$  samples always being within  $\pm 10\%$  of  
209 those of a laboratory  $\text{Ag}_3\text{PO}_4$  standard, coupled with  $\text{Ag}_3\text{PO}_4$  samples containing <0.2% carbon (based



210 on separate elemental analysis). Full replicates were processed through each stage of the extraction  
211 protocol reported in Fig. 2 on three occasions and on each occasion gave  $\delta^{18}\text{O}$  values within a range  
212 of  $\pm 0.1\text{‰}$  (see Table 2). Analytical precision (1sd) was consistently  $< 0.2\text{‰}$  and always less than  
213  $0.3\text{‰}$  (Table 2). On this basis, we consider a difference in  $\delta^{18}\text{O}_{\text{PO}_4}$  of  $\geq 0.3\text{‰}$  to be a reasonable  
214 indicator that two samples differ in isotopic composition for reasons other than analytical error.

### 215 **3. Results**

#### 216 **3.1 Inorganic chemistry**

217 Table 1 reports on-site and laboratory hydrochemical data for low flow (September 2013) and high  
218 flow (January 2014) sampling events. Temperature data reflect the climatic difference between  
219 seasons, with average water temperature  $> 11\text{ °C}$  warmer in September 2013 compared to January  
220 2014. Dissolved oxygen concentration at sites SV1 and SV2 was elevated under high flow compared  
221 to low flow conditions, consistent with temperature-related control on oxygen solubility. However,  
222 DO concentration was particularly low at SV1 under low flow conditions, suggesting either an input  
223 of strongly anoxic water to the River Beult at this site, or that there was significant consumption of  
224 oxygen upstream of SV1. Sutton Valence WwTW (SV4) delivered effluent with elevated Cl  
225 concentrations to the River Beult under both high and low flow conditions. Consistent with the very  
226 low DO concentration, Cl concentrations at SV1 under low flow conditions were elevated compared  
227 to SV2 and compared to SV1 and SV2 under high flow conditions.

228 Concentrations of  $\text{NO}_3$  and SRP at river sampling sites were generally elevated under low flow  
229 compared to high flow conditions, likely driven by reduced dilution of point sources given lower  
230 discharge (Jarvie et al., 2006). Under low flow conditions, the concentrations of SRP and other P  
231 fractions were particularly high at SV1 and SV2, whilst the concentration of  $\text{NO}_3$  at SV1 was elevated  
232 compared to that at SV2. Ammonium and  $\text{NO}_2$  concentrations were generally low at all sampling sites  
233 during both sampling events, apart from SV1 and SV2 where high  $\text{NH}_4$  and  $\text{NO}_2$  concentrations were  
234 observed under low flow conditions and, to a much reduced extent and for  $\text{NH}_4$  only, under high flow  
235 conditions. Fig. 3 reports the relationship between  $\text{NO}_3$  and Cl concentration for all sites, under both

236 low and high flow conditions ( $r=+1.00$ ,  $p < 0.01$  and  $r=+0.929$ ,  $p < 0.01$  respectively). For samples  
237 collected under low flow, a clustering of sites with high  $\text{NO}_3$  and high Cl concentrations is revealed,  
238 associated with the WwTW effluent (SV3 and SV4) and river sites downstream of the WwTW (SV5-  
239 SV7). Sites SV1 and SV2 were characterised by relatively low  $\text{NO}_3$  and Cl concentrations during this  
240 sampling event. In contrast, three clusters of sites are revealed under high flow conditions, with SV5-  
241 SV7 occupying an intermediate position between upstream sites (SV1 and SV2) and sites associated  
242 with the WwTW (SV3 and SV4). A similar clustering of sites and flow-dependency to the clustering  
243 is revealed in the relationships between SRP and Cl concentrations (Fig. 4), where  $r=-0.901$ ,  $p < 0.01$   
244 under low flow and  $r=+0.901$ ,  $p < 0.01$  under high flow.

245

246 *[Fig. 3. Nitrate concentration against chloride concentration for low and high flow sampling*  
247 *events.]*

248 *[Fig. 4. Soluble reactive phosphorus (SRP) concentration against chloride concentration for low*  
249 *and high flow sampling events (note change in scale of SRP concentration between low and*  
250 *flows).]*

251

### 252 **3.2 Stable isotope data**

253 Table 2 summarises the stable isotope dataset from the River Beult. The overall range for  $\delta^{15}\text{N}_{\text{NO}_3}$  was  
254  $+4.7$  to  $+13.1\text{‰}$ , whilst for  $\delta^{18}\text{O}_{\text{NO}_3}$  the range was  $-0.8$  to  $+8.6\text{‰}$ . Both  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  at SV1  
255 and SV2 were enriched compared to all other sites and this pattern was consistent under both high and  
256 low flow conditions. Values of  $\delta^{15}\text{N}_{\text{NO}_3}$  for SV3 and SV4 were reduced, by  $3.2$ - $3.9\text{‰}$ , during high  
257 flow compared to low flow conditions. Upstream river site SV2 was isotopically enriched compared  
258 to the final outflow from the WwTW (SV4), both for  $\delta^{15}\text{N}_{\text{NO}_3}$  (by  $2.9\text{‰}$  under low flow conditions  
259 and  $5.9\text{‰}$  under high flow conditions) and for  $\delta^{18}\text{O}_{\text{NO}_3}$  (by  $7.6\text{‰}$  under low flow conditions and  $7.2\text{‰}$   
260 under high flow conditions). Sites downstream of the WwTW (SV5-7) had  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  values

261 that were either dominated by those of the final effluent from the WwTW (low flow conditions), or  
262 were intermediate between values for SV4 and for SV1 and SV2 (high flow conditions). The  
263 relationship between  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\text{NO}_3$  concentration under low and high flow conditions is reported  
264 in Fig. 5 ( $r=-0.901$ ,  $p < 0.1$  and  $r=-0.901$ ,  $p < 0.01$  respectively). A trend of decreasing  $\delta^{15}\text{N}_{\text{NO}_3}$  with  
265 increasing  $\text{NO}_3$  concentration was observed for both sampling events, with lowest  $\text{NO}_3$  concentrations  
266 and highest  $\delta^{15}\text{N}_{\text{NO}_3}$  values occurring upstream of the WwTW. Similar trends exist for  $\delta^{18}\text{O}_{\text{NO}_3}$ ,  
267 although for brevity these data are not reported in a separate figure.

268 *[Fig. 5. Nitrate-nitrogen isotope composition ( $\delta^{15}\text{N}_{\text{NO}_3}$ ) composition against nitrate concentration*  
269 *for high and low flow sampling events. Vertical bars show standard deviation on  $\delta^{15}\text{N}_{\text{NO}_3}$  (note*  
270 *change in scale for nitrate concentration between high and low flow events).]*

271 A range of +10.0 to +33.7‰ was observed for  $\delta^{15}\text{N}_{\text{NH}_4}$ . Under low flow conditions, substantial  
272 enrichment of  $\delta^{15}\text{N}_{\text{NH}_4}$  was observed between SV1 and SV2, increasing by 12.2‰ over an in-river  
273 length of approximately 100 m. Unfortunately, the concentration of  $\text{NH}_4$  at all other sites during this  
274 sampling event was too low to enable analysis of  $\delta^{15}\text{N}_{\text{NH}_4}$ . A smaller enrichment in  $\delta^{15}\text{N}_{\text{NH}_4}$  (3.1‰)  
275 was observed between SV1 and SV2 under high flow conditions. Under high flow conditions,  $\delta^{15}\text{N}_{\text{NH}_4}$   
276 was enriched by 15.5‰ in the WwTW outflow (SV3) compared to upstream river site SV2, although  
277 insufficient  $\delta^{15}\text{N}_{\text{NH}_4}$  data were available to make this comparison under low flow conditions. At SV5-  
278 SV7 under high flow conditions,  $\delta^{15}\text{N}_{\text{NH}_4}$  was intermediate between that of sites SV2 and SV3, whilst  
279 both the concentration of  $\text{NH}_4$  and  $\delta^{15}\text{N}_{\text{NH}_4}$  remained relatively constant in the river downstream of  
280 SV4 during this sampling event.

281 The stable isotope composition of  $\text{P}_i$  varied between +15.1 and +19.0‰ across the samples. Under  
282 low flow conditions,  $\delta^{18}\text{O}_{\text{PO}_4}$  was relatively constant between SV1 and SV2. Although the absolute  
283 value of  $\delta^{18}\text{O}_{\text{PO}_4}$  was constant between these sites, the departure from the theoretical equilibrium value  
284 changed from +0.1‰ to -1.5‰ as a result of shifts in water temperature and  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  between SV1  
285 and SV2 and therefore in the theoretical equilibrium of  $\delta^{18}\text{O}_{\text{PO}_4}$  (Table 3). In contrast, under high flow  
286 conditions, the absolute value of  $\delta^{18}\text{O}_{\text{PO}_4}$  increased by 0.4‰ from site SV1 to SV2, whilst the

287 theoretical equilibrium value remained unchanged. Values of  $\delta^{18}\text{O}_{\text{PO}_4}$  at SV2 were 1.2‰ and 2.2‰  
288 enriched compared to the final effluent from the WwTW (SV4) under low and high flow conditions  
289 respectively. Under high flow conditions,  $\delta^{18}\text{O}_{\text{PO}_4}$  at sites downstream of the WwTW outflow (SV5-  
290 SV7) remained relatively constant at an average of +17.6‰  $\pm$ 0.1. Under low flow conditions, there  
291 was some evidence of decreasing  $\delta^{18}\text{O}_{\text{PO}_4}$  with distance downstream of the WwTW, although  $\delta^{18}\text{O}_{\text{PO}_4}$   
292 only decreased by 0.3‰ over the 35 m river reach and these samples remained between -0.9‰ and -  
293 1.2‰ depleted compared to the theoretical equilibrium  $\delta^{18}\text{O}_{\text{PO}_4}$  (Table 3). Marked differences in  
294  $\delta^{18}\text{O}_{\text{PO}_4}$  were observed across all sites between low and high flow conditions, with samples taken in  
295 January 2014 an average of 2‰ higher than samples taken in September 2013. The relationship  
296 between  $\delta^{18}\text{O}_{\text{PO}_4}$  and SRP concentration under low and high flow conditions is shown in Fig. 6. Under  
297 low flow conditions, there was a trend of increasing  $\delta^{18}\text{O}_{\text{PO}_4}$  with increasing SRP concentration  
298 ( $r=+0.90$ ,  $p < 0.01$ ), with the highest SRP concentration and  $\delta^{18}\text{O}_{\text{PO}_4}$  occurring at sites SV1 and SV2.  
299 In contrast, under high flow conditions the trend was reversed ( $r=-0.811$ ,  $p < 0.05$  – note lower  
300 confidence interval), with lowest SRP concentration and highest  $\delta^{18}\text{O}_{\text{PO}_4}$  values occurring in samples  
301 from these same sites.

302

303 *[Fig. 6. Stable phosphate oxygen composition ( $\delta^{18}\text{O}_{\text{PO}_4}$ ) composition against soluble reactive*  
304 *phosphorus (SRP) concentration for low and high flow sampling events. Vertical bars show*  
305 *standard deviation on  $\delta^{18}\text{O}_{\text{PO}_4}$  (note change in scale for SRP concentration between low and high*  
306 *flow)].*

307

## 308 4. Discussion

### 309 4.1 Hydrochemical insights into controls on nutrient biogeochemistry

310 The temporal and spatial variation in N and P concentrations reported in Table 1 may be interpreted  
311 through a mixing relationship between effluent from the WwTW and water in the River Beult  
312 upstream of the effluent discharge point. Under high flow conditions, WwTW effluent represents an

313 end-member with elevated  $\text{NO}_3$  and SRP concentration and is diluted on entering the river. Dilution  
314 produces downstream concentrations of  $\text{NO}_3$  and SRP in the River Beult that are intermediate  
315 between the composition of the two end-members (Figs 3 and 4). Under low flow conditions, this  
316 mixing pattern is repeated for  $\text{NO}_3$  although with reduced dilution of the WwTW effluent. However,  
317 for both SRP and  $\text{NH}_4$  under low flow conditions, the final WwTW effluent effectively dilutes  
318 enriched upstream river water, to such an extent that downstream river concentrations of  $\text{NH}_4$  and  
319 SRP predominantly reflect effluent quality (Jarvie et al., 2010; Macdonald et al., 1995). Dissolved  
320 oxygen, Cl,  $\text{NO}_3$ ,  $\text{NH}_4$  and P concentration data suggest a particular source of nutrient-enriched water  
321 influenced SV1 under low flow conditions, although these hydrochemical parameters suggest that the  
322 impact of this water source appeared to be absent, or at least significantly reduced, under high flow  
323 conditions.

324 Whilst the existence of the effluent and upstream end members, alongside flow-dependent variation in  
325 the mixing relationship between these end-members, is revealed by on-site and laboratory  
326 hydrochemical data, these data do not offer direct insights into two key questions related to  
327 understanding of the eutrophication process in aquatic ecosystems. Firstly, whilst the WwTW effluent  
328 appears to be an important source of N and P to the River Beult, the source of other nutrient inputs to  
329 the river remain uncertain, particularly at SV1 and SV2 under low flow conditions. This reflects the  
330 broader challenge of identifying the original source of nutrients, alongside the relative importance of  
331 different sources, in aquatic ecosystems (Jarvie et al., 2006). Secondly, concentration data alone do  
332 not provide direct insight into the biogeochemical mechanisms that govern the fate of nutrient  
333 elements during downstream transport within river ecosystems. Whilst indirect methods of source  
334 assessment have been developed, including the use of boron as a chemically conservative marker for  
335 WwTW effluent input to rivers (e.g. Neal et al., 2000) and microbial source tracking to identify  
336 human versus agricultural sources of faecal contamination (e.g. Scott et al., 2002), these methods do  
337 not offer an inherent label for either P or N. As a result, they lack a direct and specific means of  
338 tracing in-river transformations of these nutrients. For example, both upstream and downstream of the  
339 WwTW, concentration changes may be driven by physical mixing of water sources, by abiotic

340 geochemical mechanisms, or by metabolic processes. Discriminating between these individual  
341 processes is important if effective responses to eutrophication in aquatic ecosystems are to be  
342 developed. For example, understanding whether nutrients derived from WwTWs are strongly coupled  
343 to in-river metabolism is critical if capital and operating expenditure on nutrient removal technology  
344 at WwTWs is to be prioritised. Therefore, the extent to which a multi-stable isotope approach can  
345 provide insight into the key questions of source and in-river fate of nutrient elements is considered  
346 below.

347

#### 348 **4.2 Differentiating sources of P and N on the basis of stable isotope composition**

349 Stable isotope data can provide insight into the original sources of N and P that contribute to aquatic  
350 ecosystems. Stable isotope analyses indicate that  $\delta^{15}\text{N}_{\text{NO}_3}$  at SV1 is enriched compared to the typical  
351 composition of  $\text{NO}_3$  derived from nitrification of  $\text{NH}_4$  within soils (Kendall, 1998; Heaton et al.,  
352 2012). Instead, the  $\delta^{15}\text{N}_{\text{NO}_3}$  composition is similar to that reported for  $\text{NO}_3$  derived from sewage or  
353 from livestock slurry or manure which has undergone partial denitrification, resulting in  $\delta^{15}\text{N}_{\text{NO}_3}$   
354 between +10 and +14‰ (Anisfield et al., 2007; Kendall et al., 2007). Denitrification of these sources  
355 of N upstream of SV1 may have been responsible for the  $\delta^{15}\text{N}_{\text{NO}_3}$  composition at this site. However,  
356 whilst the dual isotopes of  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  provide a powerful basis for differentiating  
357 atmospheric and inorganic fertiliser sources of  $\text{NO}_3$  from each other, and from livestock or sewage  
358 sources, this dual isotope approach cannot distinguish between sewage and livestock sources, which  
359 requires the use of additional source markers such as boron isotopes (e.g. Briand et al., 2013).  
360 Enriched  $\delta^{15}\text{N}_{\text{NH}_4}$  at SV1 (>10‰) is also consistent with a livestock manure or slurry source that may  
361 have become isotopically enriched following volatilisation of ammonia (e.g. Widory et al., 2004).  
362 Although the existing global dataset remains relatively small,  $\delta^{18}\text{O}_{\text{PO}_4}$  at SV1 is broadly consistent with  
363 the stable isotope composition of P derived from inorganic fertiliser or livestock excreta, sources that  
364 are characterised by  $\delta^{18}\text{O}_{\text{PO}_4}$  between approximately +16 and +20‰ (see Davies et al., 2014).

365 Under low flow conditions, Cl, DO, NO<sub>3</sub>, NH<sub>4</sub> and SRP concentrations are consistent with isotopic  
366 evidence for a distinct source of N and P that influences the River Beult at SV1, likely associated with  
367 agricultural activity or unsewered households in the upstream catchment. Under high flow conditions,  
368 significant changes in these hydrochemical parameters were observed at SV1, although it is not clear  
369 whether these changes in concentration are consistent with alternative sources of N and P influencing  
370 the river under high flow compared to low flow conditions. However, stable isotope data indicate that  
371 SV1 was dominated by similar sources of N and P under both low and high flow conditions, despite  
372 substantial changes in nutrient concentration between the two sampling events. Enrichment of  $\delta^{15}\text{N}_{\text{NO}_3}$   
373 and  $\delta^{18}\text{O}_{\text{NO}_3}$ , depletion of  $\delta^{15}\text{N}_{\text{NH}_4}$  and enrichment of  $\delta^{18}\text{O}_{\text{PO}_4}$  is observed at SV1 compared to  
374 downstream river sites and compared to the effluent from the WwTW, under both high and low flow  
375 conditions. This, coupled with relatively constant  $\delta^{15}\text{N}_{\text{NO}_3}$ ,  $\delta^{18}\text{O}_{\text{NO}_3}$ , and  $\delta^{15}\text{N}_{\text{NH}_4}$  values at SV1 across  
376 both sampling events, suggests that a common nutrient source influenced SV1 in September 2013 and  
377 January 2014. The 1.9‰ shift in  $\delta^{18}\text{O}_{\text{PO}_4}$  at SV1 between low and high flow sampling events could  
378 indicate changes in the dominant source of P to the River Beult across these events. However,  $\delta^{18}\text{O}_{\text{PO}_4}$   
379 was consistent with the theoretical equilibrium value on both occasions, suggesting the 1.9‰ shift  
380 resulted from changes in equilibrium fractionation driven by changes in water temperature and  
381  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  between sampling events, rather than a change in P source. Fig. 7 shows this effect for all  
382 samples, by considering theoretical equilibrium values for a range of water temperatures.

383 *[Fig. 7. A comparison of  $\delta^{18}\text{O}_{\text{PO}_4}$  and  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  for samples collected in low flow (filled symbols) and*  
384 *high flow (open symbols). Vertical and horizontal hashed areas represent range of measured*  
385  *$\delta^{18}\text{O}_{\text{H}_2\text{O}}$  during low and high flows respectively. Diagonal dashed lines represent the  $\delta^{18}\text{O}_{\text{PO}_4}$*   
386 *equilibrium values for ambient water for the range of temperatures at low and high flows*  
387 *calculated using the equation of Chang and Blake (2015).]*

388 Under both high and low flow conditions, the stable isotope composition of NO<sub>3</sub>, NH<sub>4</sub> and SRP in  
389 final effluent samples differed substantially from that in river water upstream of the WwTW.  
390 Differentiation of nutrient sources on the basis of their stable isotope composition is the fundamental  
391 pre-requisite for using subsequent isotope fractionation to trace metabolism of nutrients derived from

392 individual sources during in-river transport. Whilst previous work has examined the stable isotope  
393 composition of  $\text{NO}_3$  and  $\text{NH}_4$  in river water and in final effluent samples from WwTWs (e.g. Sebilo et  
394 al. 2006; Hood et al., 2014), our research represents some of the first data to demonstrate  
395 differentiation between effluent and upstream river water samples in terms of  $\delta^{18}\text{O}_{\text{PO}_4}$  (although see  
396 also Gruau et al., 2005; McLaughlin et al., 2006; Young et al., 2009) The 1.4‰ shift in  $\delta^{18}\text{O}_{\text{PO}_4}$  in  
397 WwTW effluent between low and high flow conditions may indicate differences in the composition of  
398 waste water arriving at the WwTW, water residence time, or extent of metabolism within the works,  
399 and emphasises the need for more intensive characterisation and explanation of variation in  $\delta^{18}\text{O}_{\text{PO}_4}$   
400 within sources of P, such as WwTW effluent (see also Gruau et al., 2005). At SV3 under both high  
401 and low flow conditions,  $\delta^{18}\text{O}_{\text{PO}_4}$  remained -1.1‰ from theoretical equilibrium. This observation  
402 suggests that P is in excess of metabolic requirements within the WwTW, and is consistent with either  
403 an isotopically depleted source of SRP entering the works and passing conservatively through the  
404 treatment processes, or with kinetic isotope fractionation during the hydrolysis of organic P  
405 compounds within the WwTW that shifts  $\delta^{18}\text{O}_{\text{PO}_4}$  towards isotopically depleted values (Blake et al.,  
406 2005).

407

### 408 **4.3 Stable isotope evidence for the in-river fate of N and P**

409 Under low flow conditions, the concentration of  $\text{NH}_4$  decreased by two orders of magnitude between  
410 SV1 and SV2, suggesting potential nitrification within the stream network. The decrease in the  
411 concentration of  $\text{NO}_3$  between these same sites could be interpreted as evidence for in-stream  
412 denitrification or biological uptake of  $\text{NO}_3$  occurring alongside nitrification. For example, stream bed  
413 sediments have been shown to be potentially important locations for denitrification in river  
414 ecosystems (e.g. Seitzinger, 1988). Coupling stable isotope data for  $\text{NH}_4$  and  $\text{NO}_3$  enables the roles of  
415 nitrification and denitrification to be explored. Substantial increases in  $\delta^{15}\text{N}_{\text{NH}_4}$  were observed  
416 between SV1 and SV2, consistent with nitrification and a kinetic isotope effect in which isotopically  
417 lighter  $\text{NH}_4$  ions are preferentially nitrified, resulting in isotopic enrichment of the remaining  $\text{NH}_4$  in



418 the extracellular environment (Middelburg and Nieuwenhuize, 2001). However,  $\delta^{15}\text{N}_{\text{NO}_3}$  did not  
419 decrease consistently between SV1 and SV2 across both sampling events, as would be expected  
420 following generation of  $\text{NO}_3$  through nitrification (Sebilo et al., 2006). In addition, substantial  
421 decreases in  $\text{NO}_3$  concentration were observed between SV1 and SV2 under both high and low flow  
422 conditions, which is not consistent with nitrification. Whilst denitrification or biological uptake of  
423  $\text{NO}_3$  may have been responsible for decreases in  $\text{NO}_3$  concentration, no clear evidence was observed  
424 for enrichment in  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  that would be expected if denitrification or biological uptake  
425 were responsible for the decrease in  $\text{NO}_3$  concentration between SV1 and SV2 (Heaton et al., 2012).  
426 Sebilo et al. (2003) demonstrated that denitrification in stream bed sediments was not associated with  
427 a large fractionation of  $\delta^{15}\text{N}_{\text{NO}_3}$ , because diffusion of  $\text{NO}_3$  from the water column into reduced  
428 sediments is the rate-limiting, but non-fractionating, step for denitrification in these environments.  
429 However, given the three-fold decrease in  $\text{NO}_3$  concentration between SV1 and SV2 under low flow  
430 conditions, the fact that  $\delta^{15}\text{N}_{\text{NO}_3}$  actually decreased between these sites on the River Beult is not  
431 consistent with denitrification exerting a significant control on the fate of N. There does not appear to  
432 be strong isotopic evidence for denitrification within this upstream reach of the river.

433 Instead, stable isotope data suggest  $\text{NH}_4$  uptake and incorporation into biomass may have been  
434 responsible for the decreases in  $\text{NH}_4$  concentration observed between SV1 and SV2. Biological  
435 uptake under eutrophic conditions is associated with a kinetic isotope effect in which isotopically  
436 lighter ions are preferentially taken up and incorporated into biomass, resulting in isotopic enrichment  
437 of the remaining extracellular  $\text{NH}_4$  (Cifuentes et al., 1989). Whilst ammonia volatilisation may also  
438 increase  $\delta^{15}\text{N}_{\text{NH}_4}$  in any remaining  $\text{NH}_4$ , stream temperature and pH were relatively consistent  
439 between SV1 and SV2 during both sampling events, meaning that volatilisation is unlikely to have  
440 been responsible for the observed decrease in  $\text{NH}_4$  concentration between these sites.

441 The lack of any substantial change in  $\delta^{15}\text{N}_{\text{NO}_3}$  or  $\delta^{18}\text{O}_{\text{NO}_3}$  between SV1 and SV2 suggests decreases in  
442  $\text{NO}_3$  concentration were unlikely to be due to metabolism, but were instead caused by an abiotic  
443 mechanism. Given the low affinity of  $\text{NO}_3$  for sediment sorption sites, the decrease in  $\text{NO}_3$   
444 concentration alongside relatively constant  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  suggests dilution may have been

445 responsible for the decreases in  $\text{NO}_3$  concentration between SV1 and SV2. The existence of an  
446 additional input of water to the River Beult is also supported by an increase in  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  between SV1  
447 and SV2, although this was only observed under low flow conditions suggesting that groundwater  
448 discharge to the river may have been responsible for dilution of  $\text{NO}_3$  between these sites. However,  
449  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  at SV2 under low flow conditions was outside the isotopic range for groundwater in this area  
450 of the UK, which lies between +6 and +7 ‰ (Darling et al. 2003). Instead,  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  suggests a water  
451 source characterised by partially evaporated surface water influenced the river at SV2, likely from  
452 drainage systems associated with the farming activity between SV1 and SV2. Whilst dilution may  
453 also have contributed to the decrease in  $\text{NH}_4$  concentration between these sites under low flow  
454 conditions, our data indicate that changes in  $\text{NH}_4$  concentration were also associated with substantial  
455 increases in  $\delta^{15}\text{N}_{\text{NH}_4}$ . This is indicative of metabolic processes influencing the in-river fate of  $\text{NH}_4$  but  
456 not  $\text{NO}_3$ , offering insights into differences in the in-river fate of these ions as governed by their role  
457 in meeting metabolic demand for N.

458 In contrast to  $\delta^{15}\text{N}_{\text{NH}_4}$ , the value of  $\delta^{18}\text{O}_{\text{PO}_4}$  for SV1 and SV2 under low flow conditions did not  
459 change substantially, despite a decrease of almost 50% in the concentration of SRP between these two  
460 sites. Consistent with  $\text{NO}_3$ , the lack of substantial changes in stable isotope composition, coupled with  
461 a substantial decrease in concentration, suggests that an abiotic rather than metabolic mechanism  
462 controlled the concentration of SRP in this upstream reach of the river. Whilst dilution may also have  
463 been responsible for the change in SRP concentration, adsorption of  $\text{P}_i$  to stream bed sediments can be  
464 significant (Jarvie et al., 2012). Some research has suggested that the initial stages of some abiotic  
465 reactions, such as sorption, are associated with kinetic isotope effects in which isotopically lighter  $\text{P}_i$   
466 ions are preferentially removed from solution (e.g. Jaisi et al., 2010). However, we observed no  
467 evidence for this in  $\delta^{18}\text{O}_{\text{PO}_4}$  data at SV1 and SV2 under low flow conditions. Further, whilst the  
468 absolute value of  $\delta^{18}\text{O}_{\text{PO}_4}$  did not change between SV1 and SV2, the deviation from expected  
469 equilibrium  $\delta^{18}\text{O}_{\text{PO}_4}$  changed from 0.1 to -1.5‰ as a result of differences in water temperature and  
470  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  between SV1 and SV2 and therefore the theoretical equilibrium value of  $\delta^{18}\text{O}_{\text{PO}_4}$ . The  
471 increased deviation between observed and equilibrium  $\delta^{18}\text{O}_{\text{PO}_4}$  values suggests a lack of intracellular

472 metabolism of SRP in this upstream reach of the River Beult. In turn, this is consistent with molar N:P  
473 and  $\delta^{15}\text{N}_{\text{NH}_4}$  in this reach that suggest N (and specifically  $\text{NH}_4$ ) rather than P is likely to limit  
474 metabolic activity. Under high flow conditions, SRP concentration and  $\delta^{18}\text{O}_{\text{PO}_4}$  increased from SV1 to  
475 SV2. These data indicate that either an external source of P, enriched in  $\delta^{18}\text{O}_{\text{PO}_4}$ , entered the river  
476 between SV1 and SV2, or that SRP was re-generated from organic P compounds with partial  
477 inheritance of an isotopically enriched  $\delta^{18}\text{O}_{\text{PO}_4}$  composition from the source organic P compound  
478 (Blake et al., 1997; Colman et al., 2005). Molar N:P between SV1 and SV2 suggest an increased  
479 probability of P limitation or N/P co-limitation, meaning that regeneration of SRP from organic P  
480 compounds between these sites may have been promoted.

481 Examination of stable isotope data from SV5-SV7 also enables the potential links between N and P  
482 from WwTW effluent and in-stream metabolism to be examined. This is only possible because of the  
483 difference between the stable isotope composition of N and P in the effluent and in the river  
484 immediately upstream of the WwTW. With respect to  $\delta^{18}\text{O}_{\text{PO}_4}$ , strong coupling between effluent-  
485 derived P and in-river metabolism would be expected to rapidly imprint an equilibrium fractionation  
486 on SRP downstream of the WwTW, due to extensive uptake of SRP-intracellular equilibrium  
487 fractionation-release of SRP (Blake et al., 2005). Given reduced molar N:P at SV5-SV7 under low  
488 flow compared to high flow conditions, these samples are most likely to reveal isotopic evidence for  
489 in-stream metabolism of SRP. However,  $\delta^{18}\text{O}_{\text{PO}_4}$  remained approximately 1‰ away from the  
490 theoretical equilibrium along the 35 m transect downstream of the WwTW under low flow conditions,  
491 indicating little evidence for significant in-stream metabolism of effluent derived SRP in this reach.  
492 Under high flow conditions, molar N:P suggests increased potential for N limitation or N/P co-  
493 limitation at SV5-SV7. Values of  $\delta^{18}\text{O}_{\text{PO}_4}$  remained relatively constant across these sites and, on  
494 average, 0.6‰ away from the theoretical equilibrium. However, there was also little isotopic evidence  
495 for in-stream metabolism of  $\text{NO}_3$  between SV5-SV7 under either high or low flow conditions. It is  
496 likely that the constrained transect length and associated residence time provided only limited  
497 opportunity for intracellular cycling and release of SRP to imprint an equilibrium isotope fractionation  
498 on  $\delta^{18}\text{O}_{\text{PO}_4}$ , or for metabolic processes to generate fractionation in the stable isotope composition of

499 either  $\text{NH}_4$  or  $\text{NO}_3$ . Longer downstream transects should be considered in order to fully evaluate the  
500 potential links between WwTW-derived nutrients and in-stream metabolism within streams and rivers  
501 using stable isotope approaches.

502

## 503 **5. Conclusions**

504 In-situ and laboratory hydrochemical data collected from the River Beult indicate that flow-dependent  
505 changes in mixing between upstream river water and final effluent from a WwTW strongly influence  
506 downstream river nutrient concentrations. However, these hydrochemical data alone provide no  
507 insight into the original sources of nutrients that influence eutrophication risk within the river, nor into  
508 the biogeochemical processes that govern the downstream fate of these nutrients. Our research  
509 demonstrates how a multi-stable isotope framework can provide additional insights into such  
510 questions that are fundamental to understanding the eutrophication process within freshwater  
511 ecosystems.

512 Stable isotope data suggest that nutrient input to the upstream reaches of the River Beult is dominated  
513 by sewage or agricultural sources under both high and low flow conditions, despite substantial  
514 changes in nutrient concentration across these different flow conditions. Stable isotope data support  
515 the need for measures to reduce diffuse water pollution from agriculture in order to address nutrient  
516 enrichment and eutrophication risk in the upstream reaches of the River Beult. In-river changes in  
517 stable isotope composition suggest an important role for microbial uptake of  $\text{NH}_4$  to meet metabolic  
518 demands for N, particularly under low flow conditions. These data suggest that measures which target  
519 reductions in  $\text{NH}_4$  concentration within the River Beult should be prioritised in order to drive changes  
520 in autotrophic production within upstream river reaches. In contrast, changes in the concentration of  
521  $\text{NO}_3$  and SRP, interpreted through the stable isotope data, indicate that abiotic mechanisms control the  
522 fate of these ions in the upstream reach. Stable isotope data also suggest that N and P derived from a  
523 WwTW are not strongly coupled to metabolism within the river immediately downstream of the  
524 effluent discharge point, confirming the importance of addressing upstream sources of these nutrients.

525 However, further sampling along a more extensive downstream transect would be required to  
526 determine the ultimate fate of WwTW-derived nutrients within rivers using changes in stable isotope  
527 composition.

528 Three priorities for further research should be addressed in order to fully realise the potential of the  
529 multi-stable isotope framework proposed here. Firstly, the degree to which individual sources of  
530 nutrients can be reliably distinguished on the basis of their stable isotope composition requires further  
531 evaluation, particularly for  $\delta^{18}\text{O}_{\text{PO}_4}$ . Secondly, stable isotope data at higher temporal frequency are  
532 required in order to evaluate short-term changes in the isotope composition of sources, for example  
533 diurnal changes in final effluent from smaller WwTWs with short residence times. Finally, more  
534 extensive and higher-intensity spatial sampling is required to assess the in-river fate of nutrients  
535 derived from a range of sources, on the basis of changes in stable isotope composition. It is hoped that  
536 the initial evaluation of a multi-stable isotope framework for P and N reported in this paper will help  
537 stimulate future research to address these challenges.

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543

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**Table 1**  
[Click here to download Table: Table 1 final.docx](#)

**Table 1. On site parameters and concentration of selected nutrients and anions in the River Beult under low and high flow conditions.**

Site	Temp.	pH	DO	Cl	NO <sub>3</sub>	NO <sub>2</sub>	NH <sub>4</sub>	SRP	TDP	TP	N:P
	°C	-	mg/L	mg/L	mg NO <sub>3</sub> /L	mg NO <sub>2</sub> /L	mg NH <sub>4</sub> /L	µg P/L	µg P/L	µg P/L	molar
<i>Low Flow (Sep 2013)</i>											
SV1 DS Poultry and soft fruit farm	21.0	7.80	<0.5	60.1	33.4	3.11	34.0	2090	2135	2440	30
SV2 50 m ds of Livestock Farm	17.2	7.77	3.2	38.8	10.6	0.251	0.324	1296	1322	1955	3.0
SV3 WWTW outflow	20.3	7.76	6.5	93.8	131	0.126	0.043	888	918	954	68
SV4 Reed bed outflow	18.6	7.54	5.5	93.3	130	0.064	0.008	948	942	952	68
SV5 5 m ds of outflow	18.3	7.80	5.4	86.9	119	0.056	0.012	964	974	978	61
SV6 10 m ds of outflow	18.4	7.62	5.5	92.8	126	0.063	0.010	956	938	1002	63
SV7 35 m ds of outflow	18.8	7.97	7.5	91.3	123	0.024	0.028	948	956	1016	60
<i>High Flow (Jan 2014)</i>											
SV1 DS Poultry and soft fruit farm	7.4	7.71	4.5	37.7	34.5	<0.005	0.352	181	195	297	60
SV2 50 m ds of Livestock Farm	7.1	7.85	10.1	32.7	23.8	<0.005	0.332	301	322	512	24
SV3 WWTW outflow	9.6	7.60	7.9	84.2	75.1	0.232	0.105	1020	1096	1264	29
SV4 Reed bed outflow	9.8	7.20	7.4	86.1	72.1	0.014	0.025	1036	1060	1152	31
SV5 5 m ds of outflow	7.3	7.57	4.1	47.9	37.1	<0.005	0.123	516	552	706	27
SV6 10 m ds of outflow	7.4	7.51	4.2	48.6	36.9	0.100	0.134	524	559	742	25
SV7 35 m ds of outflow	6.8	7.41	4.2	46.8	35.6	0.457	0.142	524	598	768	23

**Table 2. Stable isotope data for nitrate, ammonium, phosphate and water sampled under high and low flow conditions on the River Beult. Average value reported for duplicates (<sup>a</sup>15.1 and 15.02; <sup>b</sup>16.6 and 16.65; <sup>c</sup>16.2 and 16.1). \* indicates insufficient sample to calculate standard deviation.**

Site	$\delta^{15}\text{N-NO}_3$ ‰	$\pm$	$\delta^{18}\text{O-NO}_3$ ‰	$\pm$	$\delta^{15}\text{N-NH}_4$ ‰	$\pm$	$\text{H}_2\text{O-}\delta^{18}\text{O}$	$\delta^{18}\text{O-PO}_4$ ‰	$\pm$
<i>Low Flow (Sep 2013)</i>									
SV1	13.1	0.3	7.8	0.5	10.0	0.1	-6.08	16.7	0.2
SV2	12.2	0.4	8.6	0.4	20.2	0.7	-5.22	16.6 <sup>b</sup>	0.1
SV3	8.6	0.2	-0.2	0.2	-	-	-6.84	14.7	0.1
SV4	9.3	0.2	1.0	0.2	-	-	-6.83	15.4	0.1
SV5	9.4	0.3	0.2	0.2	-	-	-6.77	15.4	0.2
SV6	9.4	0.2	-0.1	0.0	-	-	-6.78	15.2	0.1
SV7	9.5	0.4	0.2	0.3	-	-	-6.73	15.1 <sup>a</sup>	0.2
<i>High Flow (Jan 2014)</i>									
SV1	11.5	0.1	7.0	0.6	15.1	0.1	-6.50	18.6	0.1
SV2	12.0	0.1	7.4	0.1	18.2	0.4	-6.60	19.0	0.2
SV3	4.7	0.1	-0.8	0.6	33.7	*	-7.32	16.2 <sup>c</sup>	0.2
SV4	6.1	0.1	0.2	0.1	-	-	-7.34	16.8	0.1
SV5	8.9	0.1	2.1	0.2	20.2	*	-6.82	17.5	0.1
SV6	8.6	0.1	2.9	0.2	21.4	*	-6.90	17.7	0.2
SV7	8.6	0.1	3.2	0.3	19.2	*	-6.87	17.6	0.1

**Table 3. Measured temperature,  $\delta^{18}\text{O}_{\text{PO}_4}$  and  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  for water samples collected under low and high flow events on the River Beult; and the theoretical equilibrium  $\delta^{18}\text{O}_{\text{PO}_4}$  calculated using Equation 3.**

	Measured $\delta^{18}\text{O}_{\text{PO}_4}$ (‰)	Measured $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (‰)	Measured temperature (°C)	Theoretical equilibrium $\delta^{18}\text{O}_{\text{PO}_4}$ (‰)	Difference (measured- theoretical) $\delta^{18}\text{O}_{\text{PO}_4}$ (‰)
<i>Low flow (Sep 2013)</i>					
SV1	16.7	-6.08	21.0	16.6	0.1
SV2	16.6	-5.22	17.2	18.1	-1.5
SV3	14.7	-6.84	20.3	15.8	-1.1
SV4	15.4	-6.83	18.6	16.2	-0.8
SV5	15.4	-6.77	18.3	16.3	-0.9
SV6	15.2	-6.78	18.4	16.3	-1.1
SV7	15.1	-6.73	18.8	16.3	-1.2
<i>High flow (Jan 2014)</i>					
SV1	18.6	-6.50	7.4	18.5	0.1
SV2	19.0	-6.60	7.1	18.5	0.5
SV3	16.2	-7.32	9.6	17.3	-1.1
SV4	16.8	-7.34	9.8	17.2	-0.4
SV5	17.5	-6.82	7.3	18.2	-0.7
SV6	17.7	-6.90	7.4	18.2	-0.5
SV7	17.6	-6.87	6.8	18.3	-0.7



Figure 1. Location of the Beult catchment in England, UK (a) and schematic map of sample locations along a section of the river (b).

Figure 2. Schematic of the modified McLaughlin et al. (2004) protocol used to process water samples for  $\delta^{18}\text{O}_{\text{PO}_4}$  analysis.

Figure 3. Nitrate concentration against chloride concentration for low and high flow sampling events.

Figure 4. Soluble reactive phosphorus (SRP) concentration against chloride concentration for low and high flow sampling events (note change in scale of SRP concentration between low and flows).

Figure 5. Nitrate-nitrogen isotope composition ( $\delta^{15}\text{N}_{\text{NO}_3}$ ) composition against nitrate concentration for high and low flow sampling events. Vertical bars show standard deviation on  $\delta^{15}\text{N}_{\text{NO}_3}$  (note change in scale for nitrate concentration between high and low flow events).

Figure 6. Stable phosphate oxygen composition ( $\delta^{18}\text{O}_{\text{PO}_4}$ ) composition against soluble reactive phosphorus (SRP) concentration for low and high flow sampling events. Vertical bars show standard deviation on  $\delta^{18}\text{O}_{\text{PO}_4}$  (note change in scale for SRP concentration between low and high flow).

Figure 7. A comparison of  $\delta^{18}\text{O}_{\text{PO}_4}$  and  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  for samples collected in low flow (filled symbols) and high flow (open symbols). Vertical and horizontal hashed areas represent range of measured  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  during low and high flows respectively. Diagonal dashed lines represent the  $\delta^{18}\text{O}_{\text{PO}_4}$  equilibrium values for ambient water for the range of temperatures at low and high flows calculated using the equation of Chang and Blake (2015).

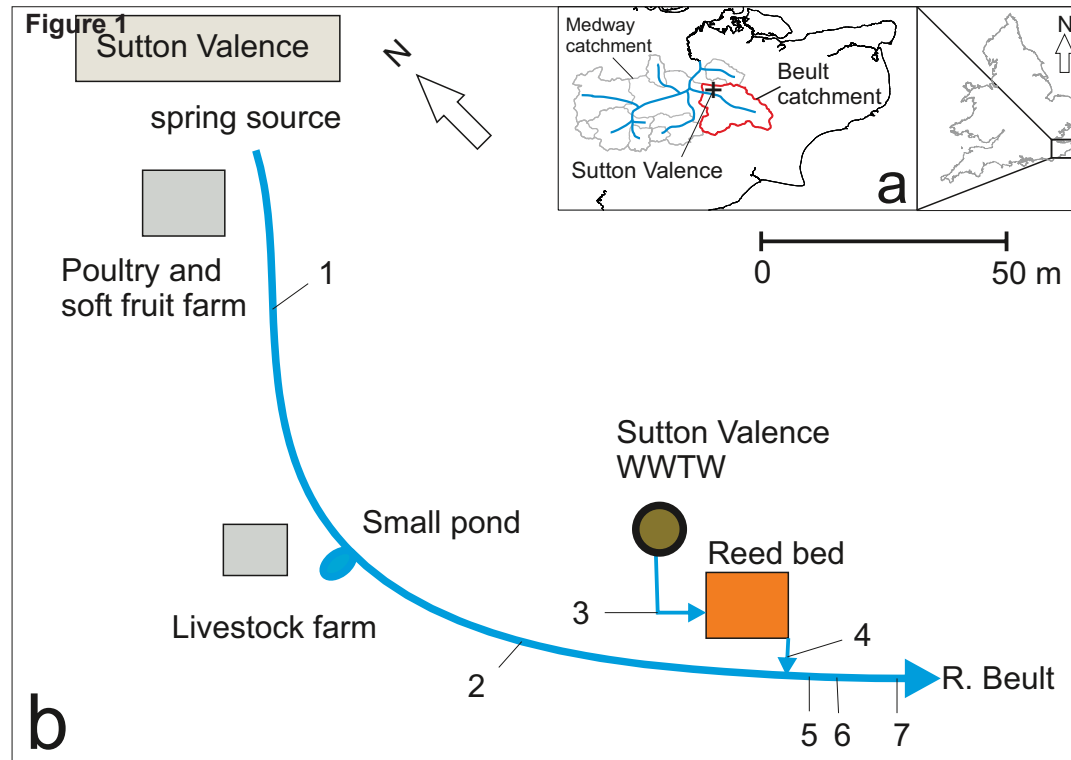


Figure 1. Location of the Beult catchment in England, UK (a) and schematic map of sample locations along a section of the river (b)

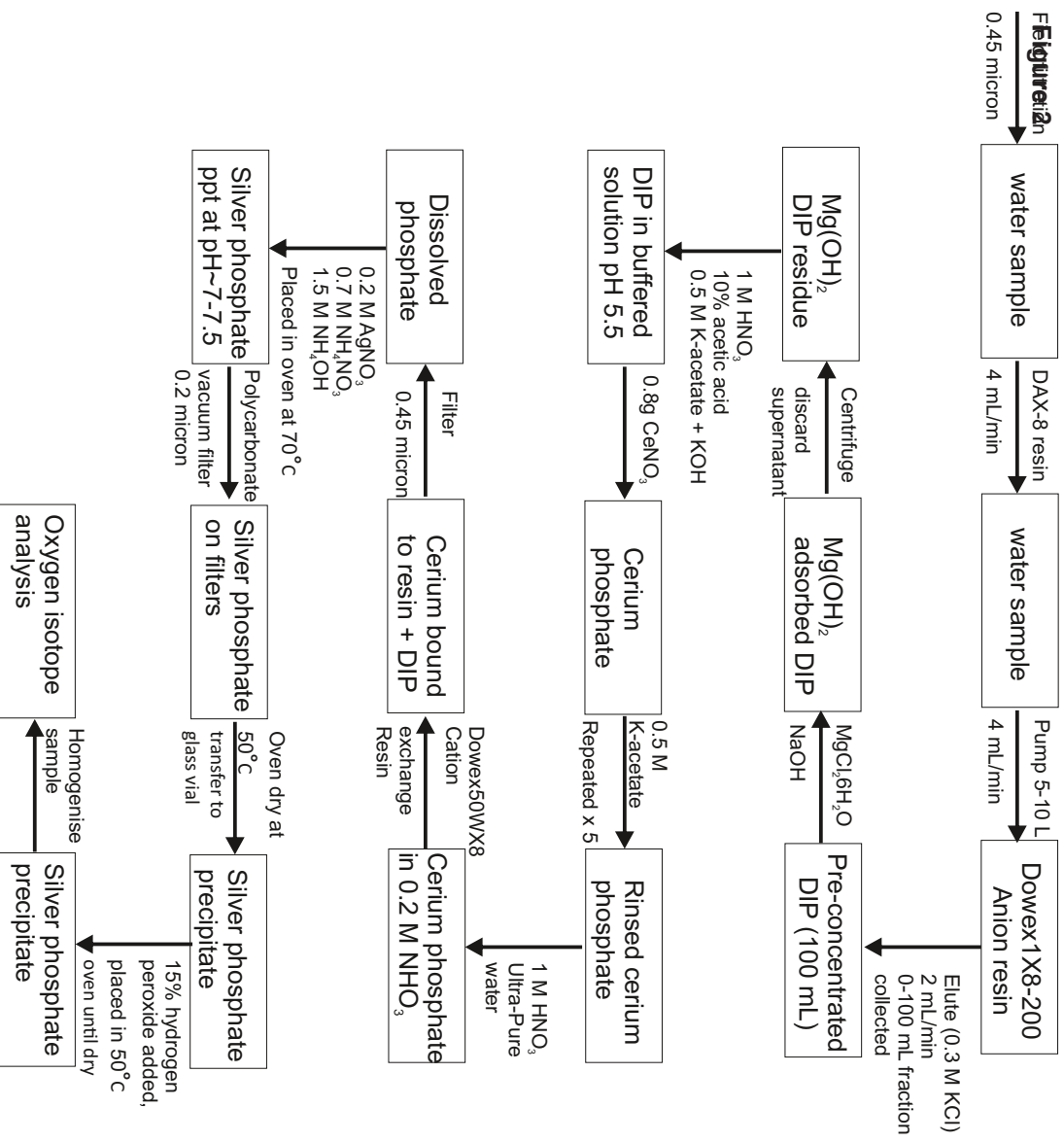


Figure 2. Schematic of the modified McLaughlin et al. (2004) protocol used to process water samples for  $\delta^{18}\text{O}_{\text{PO}_4}$  analysis.

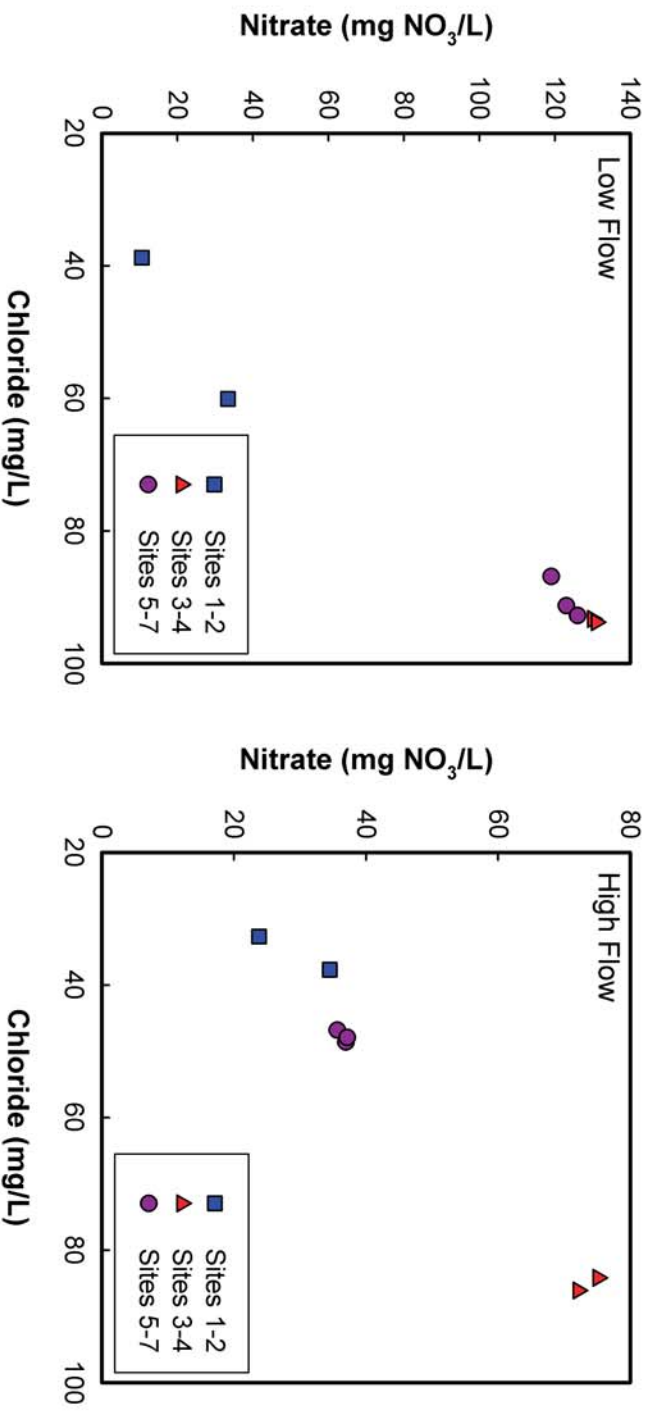


Figure 3. Nitrate concentration against chloride concentration for low and high flow sampling events.

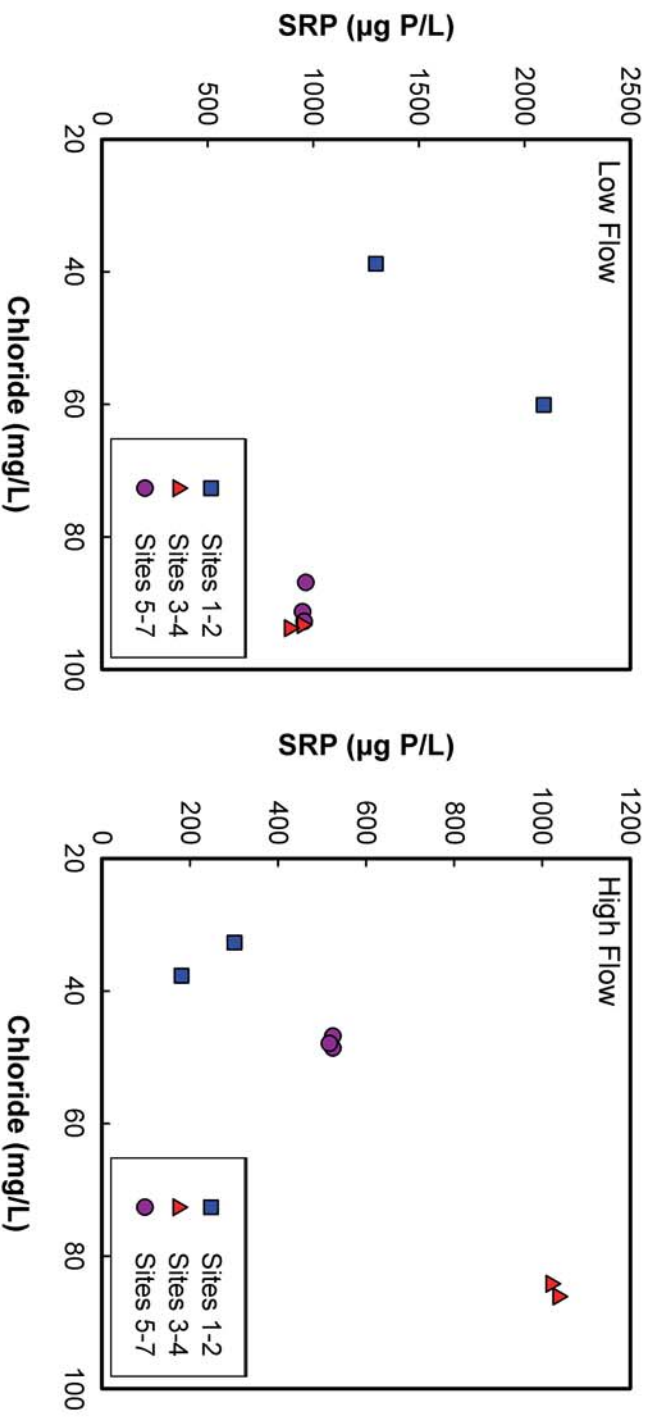


Figure 4. Soluble reactive phosphorus (SRP) concentration against chloride concentration for low and high flow sampling events (note change in scale of SRP concentration between low and flows).

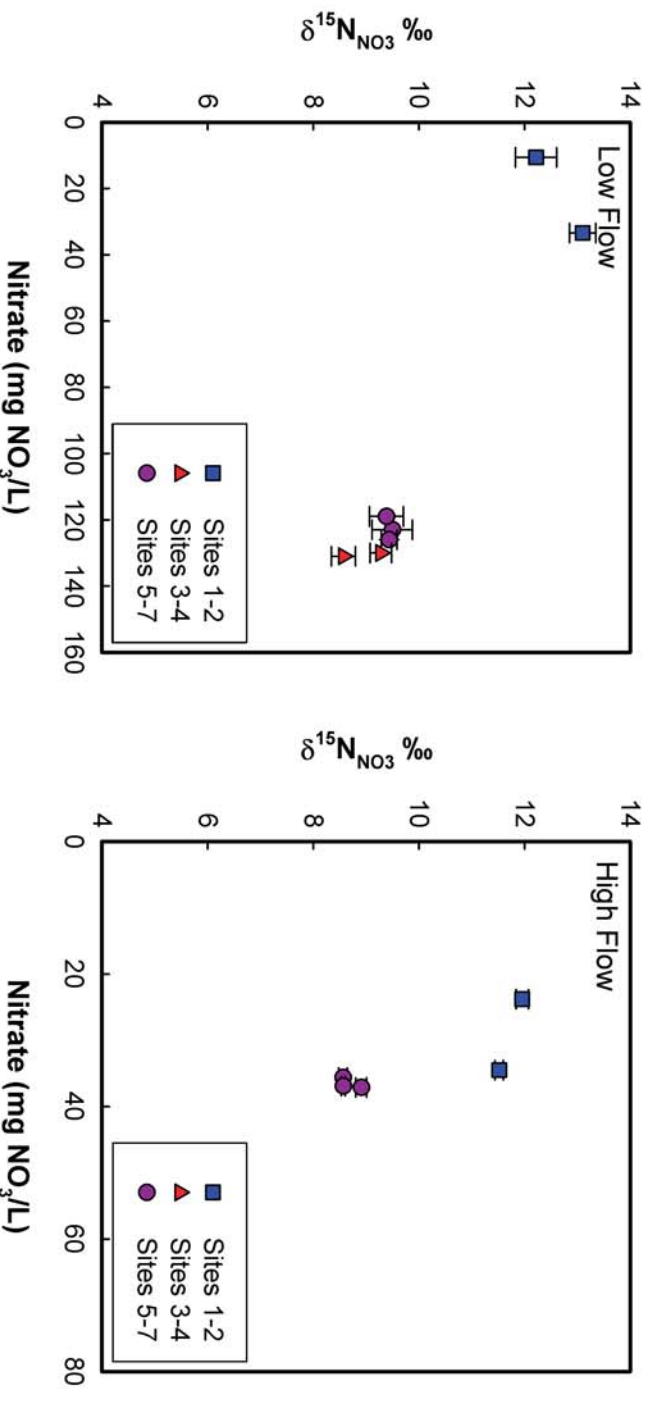


Figure 5. Nitrate-nitrogen isotope composition ( $\delta^{15}\text{N}_{\text{NO}_3}$ ) composition against nitrate concentration for high and low flow sampling events. Vertical bars show standard deviation on  $\delta^{15}\text{N}_{\text{NO}_3}$  (note change in scale for nitrate concentration between high and low flow events).

Figure 6

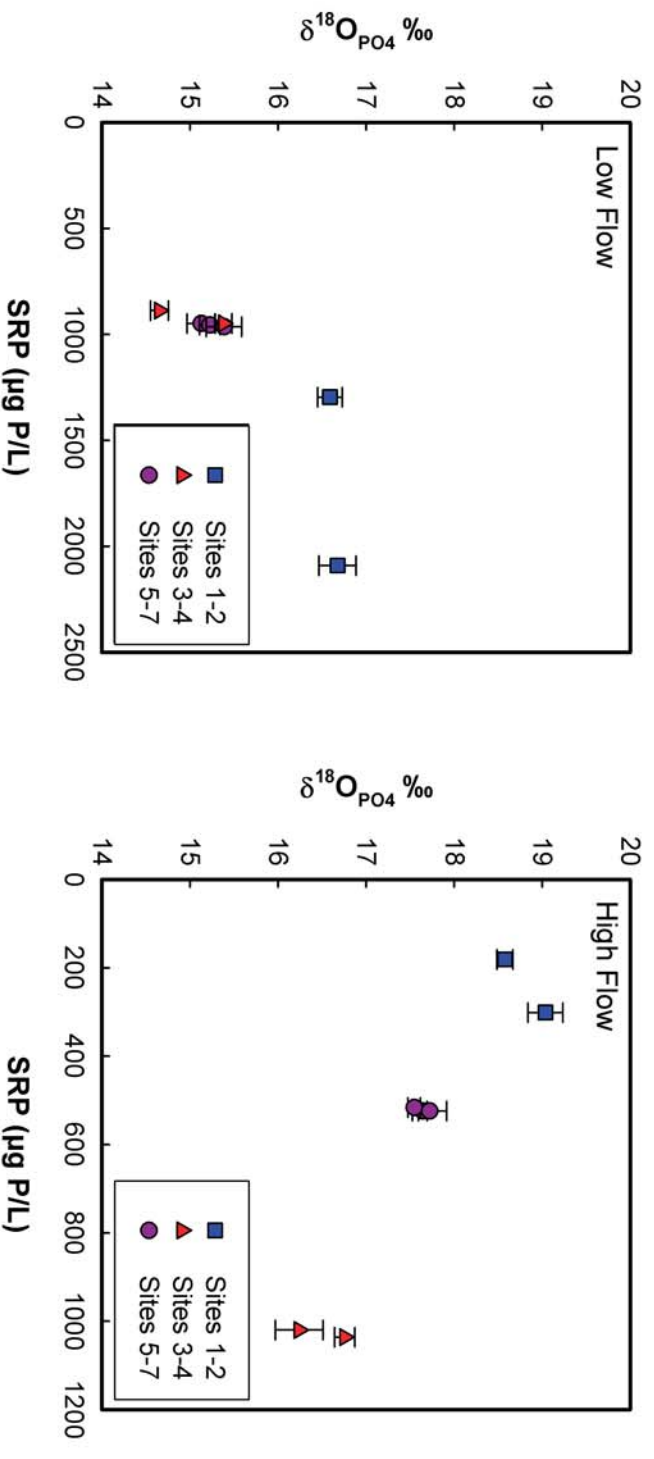


Figure 6. Stable phosphate oxygen composition ( $\delta^{18}\text{O}_{\text{PO}_4}$ ) composition against soluble reactive phosphorus (SRP) concentration for low and high flow sampling events. Vertical bars show standard deviation on  $\delta^{18}\text{O}_{\text{PO}_4}$  (note change in scale for SRP concentration between low and high flow).

