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Comparison of different methods used for phosphorus determination in aquatic organisms

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Abstract

The reliable determination of the total phosphorus (P) content stored in aquatic biota is essential for studies on nutrient stoichiometry, as well as for effective lake management measures. However, a variety of methods are found in the literature for sample P content determination, which renders it necessary to assess whether the data reported in different studies are comparable. We used different combinations of combustion durations, acid types and acid concentrations for sample digestion, and measured P concentrations subsequently with the standard colorimetric method. In addition, P contents of samples were assayed by ICP–OES and MP–AES methods. Our results confirmed that the variability among studies using different methods may explain some of the reported intraspecific and interspecific

variation. We found that duration of combustion exerted the most important influence on the P retrieval, while acid type and acidity of the hydrolysing solution did not substantially influence the efficiency of sample digestion. We recommend using 8 h of combustion and 0.3 N HCl for acid hydrolysis prior to the colorimetric P analysis, and urge standardisation in the P analyses of biotic samples so as to obtain reliable results and data comparable among different studies.

Key words: fish, benthic invertebrate, zooplankton, macrophyte, phosphorus, sample digestion

Introduction

Phosphorus (P) is a major biogenic element that often functions as a limiting nutrient in aquatic habitats, influencing primary production and ultimately total ecosystem production (Carpenter et al., 1992; Brönmark & Hansson, 2005; Dodson, 2005; Sterner, 2008). All organisms sequester and use P to support structural (e.g., bone, phospholipid and nucleic acid formation) and functional (energy transfer) demands (Sterner & Elser, 2002). However, the P content in different organisms is highly variable, being relatively low in freshwater plants (Kufel & Kufel, 2002) and the highest in fish, compared to other members of the aquatic food webs (Tarvainen et al., 2002; Frost et al., 2006; Griffiths, 2006; Boros et al., 2009). In addition, the P sequestered in different organisms is tied up in various tissues and biochemicals that differentially resist physical and chemical degradation. For instance, softer tissues like muscles may decompose and release P shortly after death, while more recalcitrant materials such as bones and scales may retain a significant fraction of their P content over several months or years (Parmenter & Lamarra, 1991; Claeson et al., 2006). This could have important implications for the dynamics of decomposition-derived internal P loading in aquatic ecosystems. In addition, and from another perspective, the presence and proportion of materials with low degradability in the bodies of aquatic organisms may determine the efficiency of whole body P content analyses.

The precise and reliable assessment of the total P content in different aquatic organisms is essential for effective and targeted lake management measures (e.g., when calculating P removal via fish or macrophyte harvesting), as well as for ecological stoichiometric analyses of aquatic food webs. However, in contrast to the more standardized carbon and nitrogen measurements − which are usually obtained by elemental analysers using the same protocol for assaying the chemical composition of samples – there are a variety of methods in the literature for P content determination, including ‘traditional’ (sample digestion and

63 subsequent colorimetric P measurement) and more modern techniques (e.g., Inductively
64 Coupled Plasma instruments). The common feature of the traditional measurements is the
65 application of the ammonium molybdate method (Strickland & Parsons, 1972) for the
66 colorimetric (spectrophotometric) quantification of the orthophosphate ions liberated after
67 various digestion procedures.

68 However, a number of different methods have been reported for sample digestion. They
69 can be divided into two main categories; (1) wet digestion of samples in acidic media (e.g.,
70 Tanner et al., 1999; 2000; Boros et al., 2009; Vrede et al., 2011); and (2)
71 combustion/incineration followed by acid hydrolysis/dissolution of the produced ash (e.g.,
72 Walve & Larsson, 1999; Sterner & George, 2000; El-Sabaawi et al., 2012). Moreover, for
73 each digestion method, we can find numerous combinations of acid types, acid
74 concentrations, and durations of heating or combustion. For example, Sterner & George
75 (2000) ashed fish samples at 500 °C for a minimum of 4 h, and subsamples of ash were acid
76 hydrolysed in 0.3 N HNO_3. Czamanski et al. (2011) followed a protocol similar to Sterner &
77 George (2000), and incinerated subsamples of whole fish homogenates and fish gut contents
78 at 500 °C for 5 h, then added 0.3 M HNO_3 to the produced ash. In addition, samples were kept
79 in tightly sealed vessels at a constant temperature of 80 °C overnight. El-Sabaawi et al. (2012)
80 also combusted fish samples at 500 °C, but they used HCl solution for acid hydrolysis at 102
81 °C for 2 h. In turn, Walve & Larsson (1999) combusted zooplankton samples at 550 °C and
82 used "persulphate solution" on a subset of their samples, and a mixture of H_2SO_4, HNO_3 and
83 H_3ClO_4, heated to 355 °C, on some other zooplankton samples. Shearer (1984) also used
84 incineration at 550 °C for fish samples, but the ash was dissolved in a mixture of equal parts
85 of concentrated HCl and HNO_3. Finally, Hendrixson et al. (2007) incinerated fish samples at
86 550 °C for 8 h and the produced ash was subsequently dissolved in 10 N H_2SO_4.

These few examples clearly demonstrate the diversity of methods used to analyse total P content of samples. It can be hypothesized that different methods vary in their efficiency in recovering P. This generates the question of whether the results of different studies on the body composition of the same species are comparable. The existing differences between studies in reported P contents (examples in Table 1) may be attributed to the natural intraspecific variability in elemental stoichiometry due to differences in the habitat, size, feeding habits, food quality or condition factor of the analysed individuals (e.g., Pilati & Vanni, 2007; Boros et al., 2012; Benstead et al., 2014), but also to differing methods.

Based on the aforementioned variability in methodology and among reported % P values, we designed the current study to compare efficiencies of the most widely applied methods for P analysis of aquatic organisms, and to reveal the comparability of body P content data reported in different studies. In addition, our aim was to find a reliable method that is relatively fast and cost-effective, and hence, could serve as a standard for body P content analyses.

Materials and Methods

Samples and sample processing

To test the reliability and efficiency of different digestion methods used prior to colorimetric P analyses, six different sample types were studied, including fish (pumpkinseed *Lepomis gibbosus* Linnaeus, family Centrarchidae; and roach *Rutilus rutilus* Rafinesque, Cyprinidae), benthic insect larvae (Diptera: Chironomidae), cladoceran zooplankton (*Daphnia* sp.) and submerged macrophyte (hornwort *Ceratophyllum demersum* Linnaeus). In addition, samples of a standard reference material (pork muscle homogenate; NCS ZC 81001) with certified 0.813±0.031 % P content were analysed to validate the measurements and test the P recoverability for each method.

112 Samples were dried to a constant weight at 60 °C and were ground to a fine powder with
113 a Retsch ZM 200 centrifugal mill. All samples (except the reference material) consisted of
114 homogenates of whole organisms. Hornwort, roach and pumpkinseed samples were collected
115 from the oligo-mesotrophic Lake Balaton (Hungary), while zooplankton and benthic
116 macroinvertebrate samples were obtained from stocks maintained as fish forage.
117
118 *Sample analysis*
119 Dried and pulverized subsamples (10−15 mg) were ashed at 550 °C for three different
120 durations (2 h, 4 h and 8 h) in 15 ml glass vials. Subsequently, the produced ash was
121 dissolved in 10 ml of 0.3 N or 1 N solution of HCl, HNO_3 or H_2SO_4, pipetted directly into the
122 glass vials after cooling. Consequently, we had 3 different variables (duration of combustion,
123 acidity, and acid type) and 18 different treatments. Each treatment consisted of three
124 replicates. After acid addition to the ashes, glass vials were capped tightly and stored at 105
125 °C for 1 h. The final step prior to colorimetric P concentration determination (Strickland &
126 Parsons, 1972) was the hundred-fold dilution of the cooled samples, resulting in a 10 ml final
127 sample volume (0.1 ml of the original solution + 9.9 ml ultrapure 'Milli-Q' water).
128 Phosphorus concentrations were measured with a Shimadzu UV 160-A spectrophotometer.
129 We also examined the effect of diluted 0.3 N and 1 N digesting acids on the outcomes of
130 colorimetric P analyses, because acidic media may affect the intensity of the blue colour
131 (proportional to the P concentration in samples) in some cases (Pai et al., 1990). To test this
132 effect, 10 ml of each acid type (0.3 N and 1 N concentrations of HCl, HNO_3 or H_2SO_4) were
133 pipetted into separate glass tubes, and were heated at 105 °C for 1h (identical to samples).
134 After cooling, a 0.1 ml subsample was taken from each tube and P concentrations were set to
135 300 μg L^{-1} in the final, 10 ml volume samples by adding 9.9 ml aqueous solution of KH_2PO_4.
136 This enabled us to see any potential deviations from the expected 300 μg L^{-1} concentration as

a function of acidity. Moreover, blank (neutral pH) samples also with 300 μg L^{-1} P concentration, consisting of KH_2PO_4 dissolved in Milli-Q water and no acids were also included.

In addition to colorimetric P content analyses, Inductively Coupled Plasma – Optical Emission Spectrometry (Agilent ICP – OES 720) and Microwave Plasma – Atomic Emission Spectrometry (Agilent MP – AES 4100) were used for P content determination on a subset of all sample types. Before ICP–OES and MP–AES measurements, dried and homogenised samples were processed with microwave digestion in Teflon vessels (0.3 g dried sample + a mixture of 5 ml 65 m/m % HNO_3 and 0.5 ml 30 m/m % H_2O_2) to liberate their total P content (Rodushkin et al., 1999; Fehér et al., 2013). The resulting solutions were diluted with ultrapure 'Milli-Q' water prior to measurements.

Statistical analyses

To explore the effect of acidity on the results of colorimetric measurements, we used the Dunnett test, wherein the concentrations measured in samples containing a mixture of standard P solution and hundred-fold diluted 0.3 N or 1 N acids (see description above) were compared to the concentrations measured in the blank samples.

The effects of combustion duration, concentration of hydrolysing solution and acid type (included as factors in the models) on the efficiency of P recovery were tested with three-way ANOVA. Subsequently, Tukey's honest significant difference (HSD) post-hoc tests were used to reveal differences between treatments, in cases where the effect of any of the factors proved to be significant ($p \leq 0.05$). Statistical analyses were performed with the StatSoft Statistica 7.0 software.

Results

Comparison of the samples containing purely hundred-fold diluted 0.3 N and 1 N acids and phosphate standard solution to the blanks showed no differences in the measurable P concentrations (Table 2). Accordingly, acidity of the diluted hydrolysing solutions was not found to influence the results of colorimetric measurements, which means that neutralisation of samples could be omitted during analyses.

The positive effect of increased combustion duration on the measurable P concentrations was obvious in all sample types, being the most pronounced in the case of pumpkinseed samples (Fig. 1). Here, the difference between the lowest (2 h, 0.3 N HCl treatment) and the highest (8 h, 1 N HCl treatment) measured % P values was more than 21%. The second largest difference (18.2 %) between the lowest (2 h, 0.3 N H_2SO_4) and highest (8 h, 1N H_2SO_4) % P values occurred in roach samples. Moreover, for pumpkinseed, roach and hornwort samples, there was virtually no overlap between the results obtained by colorimetric methods (including all digesting treatments) and those by ICP–OES and MP–AES. In contrast, there was considerable overlap between the results obtained by ICP–OES and colorimetric measurements for benthic macroinvertebrates, zooplankton, and the reference material. However, for all sample types, measurements with MP–AES produced consistently lower % P values than other methods.

ANOVA revealed that combustion duration was the only factor influencing the efficiency of digestion for all samples types (Table 3). Acid concentration was significant only for benthic macroinvertebrate samples, while the type of acid did not affect the efficiency of digestion for any samples.

As combustion duration proved to be the most important factor in determining the P yields from all sample types, the three different durations (2 h, 4 h, 8 h) were compared to assess significant differences between treatments and the time interval that is required for

187 effective sample digestion. We found that 2 h of combustion was not sufficient for the
188 efficient sample decomposition. In turn, 4 h of combustion was sufficient in the case of roach,
189 hornwort and reference material samples, while 8 h of incineration yielded significantly
190 higher P contents in pumpkinseed, benthic macroinvertebrate and zooplankton samples (Fig.
191 2).
192
193 **Discussion**
194 Our results suggest that the reported among-study variation in P contents may be
195 explained at least in part by methodological inconsistencies. It was found that the duration of
196 combustion exerted the most important effect on sample decomposition and thus on the
197 efficiency of P retrieval. Even though 2 hours of incineration prior to acid hydrolysis is not
198 commonly used in P content determination of biotic samples, we decided to test the efficiency
199 of this relatively short time interval, because we assumed that for some easily degradable
200 sample types, 2 hours at 550 °C may be sufficient. This could save time and energy during
201 analyses. However, our results show that samples must be combusted for at least 4 hours to
202 obtain reliable results on P content. Nevertheless, using 8 hours of combustion was the most
203 effective among the methods we compared. In contrast, acid type and the acidity of the
204 hydrolysing solution did not influence the efficiency of digestion considerably, and
205 consequently all of the acid combinations we used in this study are eligible for sample
206 digestion and would be expected to produce comparable results. Moreover, the results
207 highlight that if samples contain hundred-fold diluted 0.3 N or 1 N acids, neutralisation prior
208 to colorimetric measurements is not necessary, which could accelerate and simplify the
209 process of P content determination.
210 Different sample types contain recalcitrant components in different proportions, and the
211 results suggest that 4 hours of incineration may not be able to degrade all particles and

212 molecules that bind P in benthic insect, zooplankton and pumpkinseed samples. The
213 difference between roach and pumpkinseed in the duration necessary for effective
214 decomposition could be attributed to the different anatomy of the two species. The proportion
215 of bony matter is higher in the bodies of centrarchid fish (pumpkinseed), compared to
216 cyprinids (roach) (Hendrixson et al., 2007). Bones, scales and other hard structures store 73 –
217 88 % of the total P content in teleost fish body (Rønsholdt, 1995; Hendrixson et al., 2007),
218 and these tissues resist rapid degradation under natural decomposition (Parmenter & Lamarra,
219 1991; Claeson et al., 2006), and probably act as the most recalcitrant materials during
220 laboratory digestion as well. Likewise, for benthic macroinvertebrates and zooplankton, 8
221 hours of combustion yielded the highest P contents, most probably due to the presence of
222 recalcitrant materials such as P embedded in chitinous structures. It is assumable that 8 h of
223 combustion is sufficient for effective sample decomposition for all biotic samples, but further
224 exploration is needed to verify this, including samples from a wide range of aquatic and
225 terrestrial taxa.

226 Surprisingly, P contents assayed with ICP–OES, and particularly with MP–AES, were
227 typically lower than those obtained through colorimetric measurements. Thus, MP–AES is
228 likely to underestimate the actual P content in all sample types (except for the reference
229 material), while ICP–OES measurements resulted in rather low P values in fish samples, but
230 not in benthic macroinvertebrates and zooplankton. We presume that the relatively low P
231 recoveries obtained with ICP and MP methods may be attributed to the lower efficiency of
232 sample digestion that was used prior to these measurements. However, we followed a
233 digestion protocol that is normally used before ICP and MP measurements (Rodushkin et al.,
234 1999; Fehér et al., 2013). Moreover, the consistent differences between the results obtained by
235 ICP and MP methods may be the consequence of their dissimilar sensitivity in detecting P.
236 These results suggest that microwave digestion with acids in Teflon vessels is only

moderately effective for some sample types, and this is especially true for fish samples, which store most of their P in heavily recalcitrant bone and scale fragments. This finding draws attention to the need for some refinement in the methodology of sample preparation used before ICP and MP measurements.

Various methods for sample digestion prior to colorimetric P concentration determination can be found in the literature, including different combinations of incineration duration, acid concentration and acid type. We have established that acid type and acidity of the hydrolysing solution do not significantly affect the P recovery. Thus, we presume that % P values reported for a particular species in different studies are comparable to each other, when different acids were used for sample acid hydrolysis. However, the use of variable combustion durations may render it difficult to compare the reported P contents in some cases. In fact, the differences between P values obtained by different methods from the same species are comparable to the natural interspecific variations. For instance, Czamanski et al. (2011) established that farmed rainbow trout (*Oncorhynchus mykiss* Walbaum) have 1.3 % body P content (in dry mass), while Hendrixson et al. (2007) reported 2.4 % P on the same species, collected from an oligotrophic lake. These studies differed in their combustion duration: Czamanski et al. (2011) used 5 h of combustion, while Hendrixson et al. (2007) incinerated samples for 8 h. Moreover, studies differed in the combustion temperature (500 *vs*. 550 °C) and in the acids used for dissolving the produced ashes (0.3 M HNO_3 *vs*. 10 N H_2SO_4). We have to note that farmed rainbow trout (Czamanski et al., 2011) had higher (56.7 %) body carbon content, compared to wild-caught rainbow trout (47.5 %; Hendrixson et al., 2007), which might contribute significantly to the remarkable differences in P contents, because any changes in the proportion of carbon may drive ("dilute") the relative proportions of most other elements, including P. However, the methodological dissimilarities may explain at least a fraction (8 – 10%) of the among-study variation in %P contents, which is also important. Thus, we suggest

and urge the international standardisation in P content analyses of biotic samples, to eliminate variability that may arise from the various and in some cases unpredictable efficiency of different methods used for determining sample P contents.

Conclusion

We recommend using 8 h of incineration before acid hydrolysis of samples for P analysis, as this duration was proven to be the most effective among the methods we compared. Because there were no considerable differences between acids in their digesting efficiency, we suggest using 0.3 N HCl for acid hydrolysis, as this method was the most cost-effective in our study. By implementing the same protocol during P analyses, results published by different authors would be more reliably comparable, thereby facilitating comparison of the actual variation in elemental composition arising from ecological and environmental factors.

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356

357

Tables

Species	*Reported % P content	Reference
Rainbow trout (*Oncorhynchus mykiss* W.)	1.3	Czamanski et al., 2011
	2.4 ± 0.4	Hendrixson et al., 2007
Brown bullhead (*Ameiurus nebulosus* L.)	2.6 ± 0.6	Tanner et al., 2000
	3.4 ± 0.4	Hendrixson et al., 2007
Northern pike (*Esox lucius* L.)	2.1 ± 0.3	Tanner et al., 2000
	3.5 ± 0.2	Hendrixson et al., 2007
Golden shiner (*Notemigonus crysoleucas* M.)	2.7 ± 0.3	Tanner et al., 2000
	3.5 ± 0.3	Hendrixson et al., 2007

*Mean % P values in dry mass ± SD (where available)

Table 1: Reported % P values of four different fish species from the literature. The variability between studies, in which different methods were used for assaying the P content of samples, is illustrated.

HCl 0.3N		HCl 1N		HNO_3 0.3N		HNO_3 1N		H_2SO_4 0.3N		H_2SO_4 1N	
%	p	%	p	%	p	%	p	%	p	%	p
99.62	0.219	99.82	0.851	99.56	0.154	99.79	0.765	100.08	0.958	100.11	0.987

373

374 Table 2: Comparison of the P concentrations measured in diluted acid solutions and blank
375 samples, revealing no significant differences. Percentages indicate the recoverability of P
376 concentration measured in the blanks, while "p" denotes the significance of difference
377 between P contents measured in the acid solutions and blanks.

378

379

380

	Combustion duration		Acid type		Acid concentration	
	$F_{2,48}$	p	$F_{2,48}$	p	$F_{1,48}$	p
Pumpkinseed	18.029	0.000	2.300	0.111	0.687	0.411
Roach	7.229	0.002	0.283	0.755	3.401	0.071
Benthic macroinvertebrate	85.500	0.000	0.894	0.416	39.929	0.000
Cladoceran zooplankton	51.783	0.000	2.547	0.089	2.217	0.143
Hornwort	45.311	0.000	2.089	0.135	1.065	0.307
Reference material	30.540	0.000	0.674	0.514	1.927	0.171

381

Table 3: The effect of the three factors (combustion duration, acid type and acid concentration) on P recovery. The ANOVA showed that combustion duration was the only significant factor, whereas the effects of acid type and acidity of the hydrolysing solution were not significant (except in the case of acid concentration for benthic macroinvertebrates).

Figure captions

Fig. 1: Various P recoveries as a function of combustion duration, acid type and acid concentration for the different sample types. Each point represents average ± SD values. Dashed lines: the P concentration assayed with ICP–OES; dotted lines: the P concentration assayed with MP–AES; continuous line (last plot): the certified value (CV) of the reference material

Fig. 2: Efficiencies of different combustion durations (2 h, 4 h, 8 h) in recovering the P content from various sample types. Lower case letters above the boxes denote the similarity/difference of treatments (treatments denoted with the same letter do not differ significantly; $p \geq 0.05$)

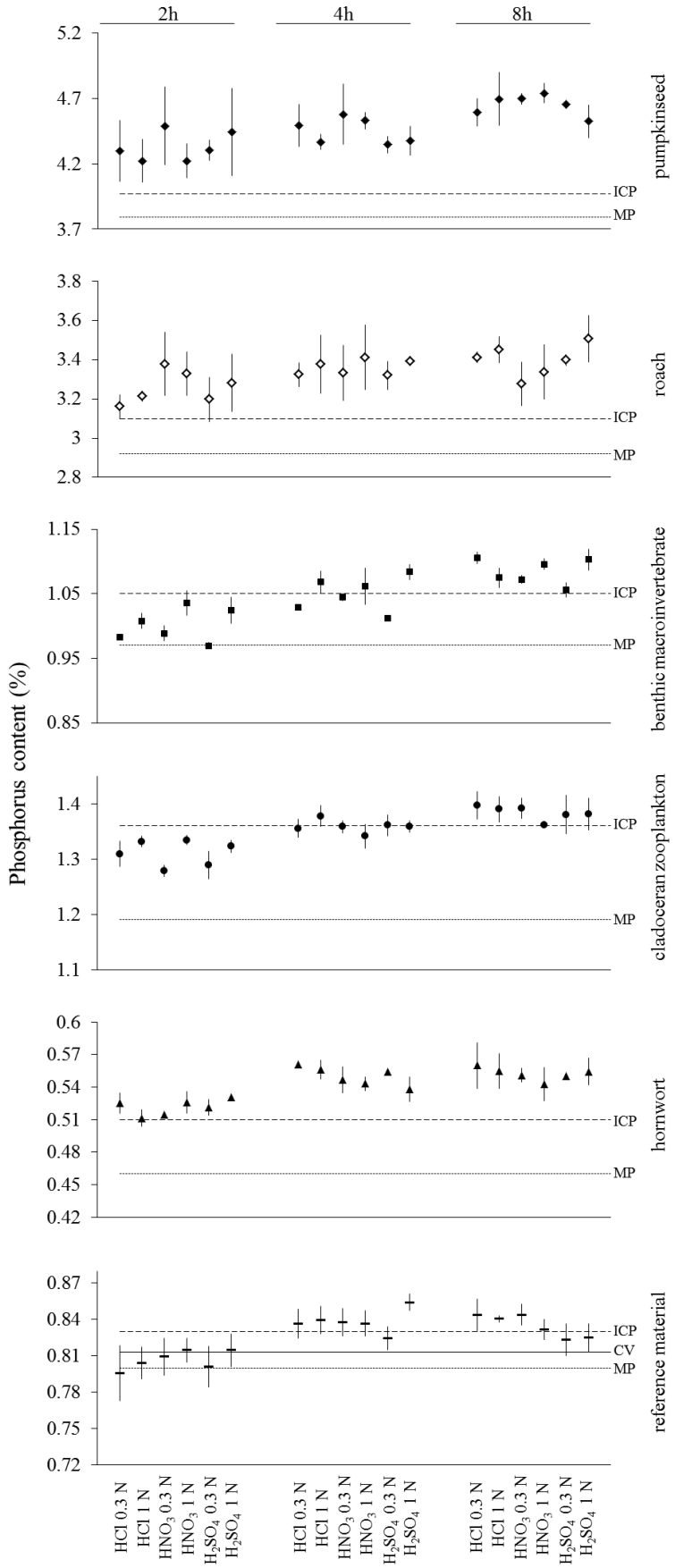
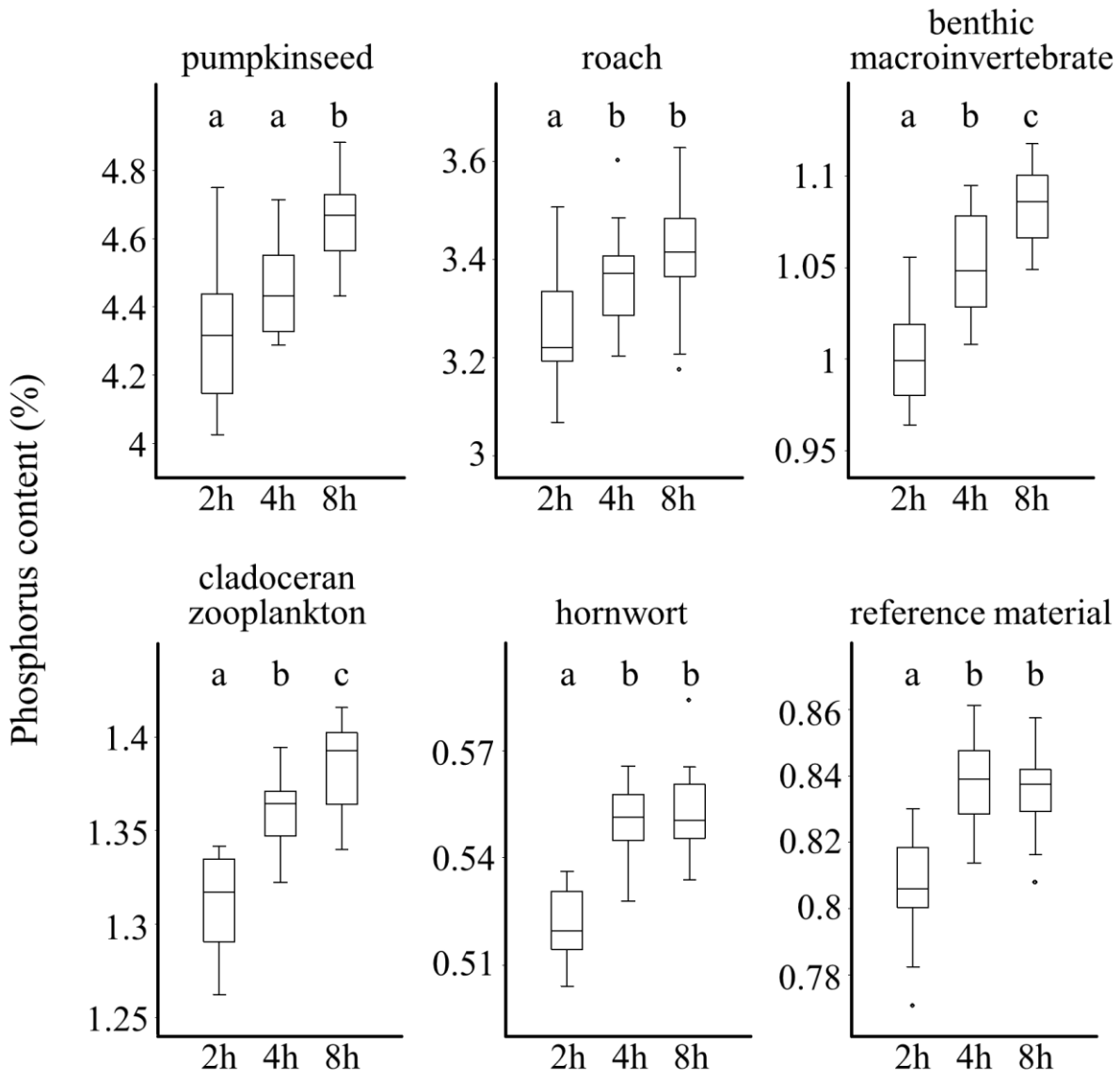

2h
4h
8h
Phosphorus content (%)
pumpkinseed
roach
benthic macroinvertebrate
cladoceran zooplankton
hornwort
reference material
ICP
MP
CV
HCl 0.3 N
HCl 1 N
HNO3 0.3 N
HNO3 1 N
H2SO4 0.3 N
H2SO4 1 N

Fig. 1



403

404 **Fig. 2**

405