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Effect of Storage Method and Associated Holding Time on Nitrogen and Phosphorus Concentrations in Surface Water Samples

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Abstract Assessments were conducted to determine the effect of sample storage method and associated holding time on surface water nutrient concentrations from field sites. Six surface water sites and two nutrient spiked, laboratory water samples were evaluated for nitrate, nitrite, ammonium, filtered orthophosphorus, and total orthophosphorus concentrations on four separate days throughout the period of 1 year. Samples stored at ambient temperature (23°C) for 24 h prior to nutrient analyses resulted in 18 % ± 2 % of results being significantly different from controls (which were analyzed immediately upon collection). Samples placed in the cooler (4°C) for 7 days prior to nutrient analyses resulted in 30 % ± 1 % of values being significantly different from controls. Samples placed in the freezer (−20°C) for 7 days prior to analyses resulted in 34 % ± 12 %, 44 % ± 10 %, and 28 % ± 5.7 % of ammonium, filtered orthophosphate, and total orthophosphate, respectively, values being significantly different from controls. This study highlights the challenges facing researchers in efficient collection, storage and nutrient analysis of samples, especially when sites are remote and difficult to access .

Keywords Nutrients · Water quality · Freezing · Refrigeration · Ambient temperature

Reliable water quality data are essential when addressing significant issues facing our Nation's waters, such as hypoxia in the Gulf of Mexico. Many challenges exist, such as when, where and how many samples to collect in a given water body to derive the most accurate results. For nutrient samples, US Environmental Protection Agency (EPA) protocols recommend acid preservation, immediate cool (4°C) storage, and analyses within 48 h (US EPA 1983). However, due to the remoteness of sampling sites in some locations, cool storage within quality assurance plan parameters is not always possible (Burke et al. 2002).

In addition to sample collection challenges, issues also arise about sample preservation and storage methods. Gardolinski et al. (2001) pointed out the difficulties in selecting a lone sample preservation method because of variability in physicochemical parameters. Other factors including sample matrix, filtration techniques, and storage container type and size can affect the preservation method (Gardolinski et al. 2001). Acidification has been reported to affect both orthophosphate and nitrite (NO_2) sample integrity in Chesapeake Bay water samples (Salley 1995). Kopsky et al. (2010) noted that although acidification can extend holding times of samples with high nutrient concentrations, it may negatively alter samples with low nutrient concentrations.

Although freezing is used as a preservation method by many researchers (Triska et al. 1989; Mitchell and Lamberti 2005), nutrient surface water concentrations can substantially decrease if analyses are conducted with high temperature combustion or ion chromatography (Fellman et al. 2008). If sample storage is on the hours to days timescale, refrigeration at 4°C is a preferred method (Jarvie et al. 2002). Fishman et al. (1986) reported that refrigeration at 4°C with no other preservation method was adequate for 8 days storage of aqueous nutrient samples.

Given the various challenges and factors involved in sample storage methods, it is critical to develop a suitable

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protocol which will minimize the physical, biological, and chemical processes that may alter sample nutrient concentrations. The objective of the current study was to examine if significant differences existed between different storage methods (ambient temperature for 24 h; cooler at 4°C for 7 days; and freezer at −20°C for 7 days) and resultant nitrogen and phosphorus concentrations when compared to those sample replicates immediately analyzed.

Materials and Methods

Surface water samples from six sampling sites were collected on four separate dates (January 4, 2010; April 12, 2010; October 18, 2010; and January 26, 2011) for the experiment. Each sampling date included two additional "sites," which were laboratory spikes prepared using Milli-Q^{TM} deionized water, for a total of eight samples. The six field sample sites were all within a 32 km radius of the US Department of Agriculture-Agricultural Research Service's National Sedimentation Laboratory (USDA-ARS NSL) to minimize transport time.

Two of the six field sites (sites 1 and 2) were located on Toby Tubby Creek, part of a 121.5 km^2 watershed which drains urban, commercial, and wooded lands in Lafayette County, Mississippi. The creek rises on the northwest side of the city of Oxford, flows southwesterly past the USDA-ARS NSL, then westerly to its confluence with Sardis Reservoir. From 1992 to 2004, the USDA-ARS NSL collected monthly or bimonthly surface water samples at these locations for routine water quality analyses as part of the Demonstration Erosion Control (DEC) Project in north Mississippi.

Two field sites (sites 3 and 4) were located on the property of the University of Mississippi's Field Station (UMFS). Two experimental spring-fed ponds were chosen from across the 300 ha site of more than 200 ponds in Lafayette County, Mississippi, approximately 18 km northeast of the University's campus in Oxford, Mississippi.

The two remaining field sites (sites 5 and 6) were located on Burney Branch Creek, whose watershed drains approximately 41.5 km^2 in central Lafayette County, Mississippi, south of the city of Oxford. Headwaters are within the city limits and the stream flows south some 8.9 km to its confluence with the Yocona River. Commercial development occupies 25 % of the watershed's area, and as with Toby Tubby Creek, the USDA-ARS NSL began monthly surface water quality monitoring on these sites in 1992, continuing until 2004 as part of the DEC Project.

As stated earlier, two laboratory spikes (sites 7 and 8) rounded out the eight sampling "sites". Laboratory spikes were prepared with Milli-Q^{TM} deionized water, along with nitrate (NO_3) (as $NaNO_3$, Fisher Scientific), ammonium (NH_4) [as $(NH_4)_2SO_4$, Fisher Scientific], and orthophosphate (PO_4) (as K_2HPO_4, Fisher Scientific) prepared stocks for a target of 3 mg L^{-1} for each main constituent.

With the exception of the January 4, 2010 sampling, three replicate 250 mL polyethylene cups were used to collect water for nutrient analyses for each different storage method. Only two replicate cups were collected on the initial sampling day of January 4, 2010. All samples were placed on ice and returned to the laboratory within 2 hours of initial collection.

On each sampling day, all samples were returned to the laboratory where one set was run immediately (within 4 h of collection; serving as a control); a second set was placed immediately in the freezer (−20°C) for 1 week before being removed, thawed, and immediately analyzed; a third set was put in the cooler (4°C) immediately for 1 week before being removed and analyzed; and a fourth and final set was allowed to sit at ambient temperature (23°C) for 24 h before being analyzed.

All water samples were analyzed for NO_3, NO_2, NH_4, total orthophosphate (TOP), and filtered orthophosphate (FOP). Briefly, NO_3 was determined using the cadmium reduction method (Hach Method 8192), whereas NO_2 was determined using the USEPA Diazotization Method 8507. Ammonia was analyzed using the phenate method, FOP was determined using the ascorbic acid method, and TOP was analyzed according to the persulfate digestion method. All of which were based on standard methods (Murphy and Riley 1962; APHA 2005). Detection limits for NO_3, NO_2, and NH_3 were 0.063 mg L^{-1}, while FOP and TOP had detection limits of 0.094 mg L^{-1}.

Descriptive statistics were used to evaluate data, while statistical significance between the control and sample storage methods were evaluated using JMP® 8.0.1 statistical software and Student's *t* tests with an alpha level of 0.05. Determinations of statistical significance among sample storage methods utilized the same software with ANOVA and Tukey's Honestly Significant Difference test with an alpha level of 0.05.

Results and Discussion

Overall, 18 % ± 2 % of samples left at ambient temperature for 24 h differed from controls when analyzed for all nutrients. Of those samples immediately placed in the cooler or freezer for 1 week, 30 % ± 1 % and 32 % ± 4 %, respectively, differed from controls. When evaluating each of the storage methods against specific nutrient analyses, samples left at ambient temperature

Table 1 Overall mean percentage (±SE) of samples (based on mean percentages from 8 individual sites) stored under varying conditions that differed from controls with respect to concentrations of various nutrients

Analysis	Mean percentage ± SE		
	Ambient	Cooler	Freezer
Nitrate	6.3 ± 4.1^{a}	38 ± 4.7^{b}	13 ± 6.7^{a}
Nitrite	16 ± 6.6^{a}	38 ± 6.7^{a}	41 ± 8.1^{a}
Ammonium	19 ± 6.3^{a}	28 ± 8.8^{a}	34 ± 12^{a}
Filtered orthophosphate	25 ± 9.5^{a}	34 ± 4.6^{a}	44 ± 10^{a}
Total orthophosphate	22 ± 3.1^{a}	13 ± 4.7^{a}	28 ± 5.7^{a}

Values in rows not connected by the same letter are significantly different from each other based on ANOVA and Tukey's HSD tests (n = 112 for each storage method and analysis)

Ambient: held 24 h at 23°C

Cooler: held 7 days at 4°C

Freezer: held 7 days at −20°C

(with the exception of TOP analyses) had the fewest significant differences from controls with regard to nutrient concentrations (Table 1). It is possible that the time until analyses (24 h vs. 7 days) played a role in these results, even though no chemical preservation was used.

The percentage of NO_3 analyses that differed from the control was significantly greater in cooler samples than in both ambient ($p = 0.0012$) and freezer samples ($p = 0.0082$). Kopsky et al. (2010) reported frozen samples with medium (1.44–1.47 mg L^{-1}) to high (23–26 mg L^{-1}) NO_3 concentrations were relatively stable over 28 days. Current field sample values for NO_3 were less than those analyzed by Kopsky et al. (2010), ranging from 0.11 to 1.07 mg L^{-1}. Current spiked samples ranged from 5.93 to 11.4 mg L^{-1}, falling between the medium and high range reported by Kopsky et al. (2010). For NO_2, all storage methods resulted in the same magnitude of variation with respect to the controls (average of 32 % differed from controls regardless of storage method). For NH_4, all storage methods resulted in the same magnitude of variation with respect to the controls (average of 27 % differed from controls regardless of storage method). Kopsky et al. (2010) recommended, however, that neither refrigeration nor freezing would extend the holding time of samples to be analyzed for NH_4. Additionally, they recommended samples should be analyzed within 48 h, which differs from US EPA's allowance of a 28 days holding time (Kopsky et al. 2010). Vesley (1990) demonstrated that precipitation and lake water samples stored at 4°C showed significant changes in NH_4 concentrations after just 24 h of storage. As with NH_4, all storage methods resulted in comparable variation in FOP concentrations with respect to the controls (average of 34 % samples differed from the controls regardless of storage method) (Table 1). The highest number of total samples that were different from controls when analyzed for FOP was from those stored in the freezer. Fellman et al. (2008) observed a significant decrease in total dissolved phosphorus concentrations when samples were frozen. For TOP, samples stored in the cooler resulted in the lowest percentage differing from the control, while those samples which were frozen resulted in the highest percentage (Table 1). Transformation of phosphorus species in samples can occur, whether stored short- or long-term, resulting in either elevated or decreased concentrations in various measured fractions (Jarvie et al. 2002).

Within individual sampling locations, significant differences in annual mean NO_3 concentrations existed. At site 1, significant differences existed between the control and cooler samples ($p = 0.0408$), as well as between the cooler and freezer samples ($p = 0.0310$). Significant differences between the control and cooler samples were also noted in sites 2 and 3 ($p = 0.0329$ and $p = 0.0247$, respectively). At site 6, significant differences existed between control and ambient samples ($p = 0.0480$). In both spiked samples (sites 7 and 8), significant differences were noted between the control and cooler samples ($p = 0.0356$, $p = 0.0221$) and between the cooler and freezer samples ($p = 0.0452$, $p = 0.0108$). Reported annual mean NO_3 concentrations (excluding spike sample sites 7 and 8) ranged from 0.12–0.80, 0.11–0.85, 0.31–1.07, to 0.13–1.03 mg L^{-1}, in control, ambient, cooler, and freezer samples, respectively (Table 2).

For annual mean NO_2 concentrations, significant differences existed in sites 6, 7, and 8. For site 6, significant differences were noted between control and cooler samples ($p = 0.0024$). At site 7, significant differences existed only between control and freezer samples ($p < 0.0001$). Significant differences at site 8 were present between control and freezer ($p < 0.0001$), cooler and freezer ($p < 0.0001$), and ambient and freezer samples ($p < 0.0001$). Excluding spike sample sites 7 and 8, reported annual mean NO_2 concentrations ranged from 0.003–0.008, 0.002–0.009, 0.005–0.012, to 0.004–0.009 mg L^{-1}, in control, ambient, cooler, and freezer samples, respectively (Table 3).

Significant differences in annual mean NH_4 concentrations existed at four individual sampling sites when comparing controls, ambient, cooler, and freezer samples (Table 4). Site 2 expressed significant differences between control and freezer samples ($p = 0.0287$). For site 3, significant differences were noted between control and cooler ($p = 0.0049$), control and freezer ($p = 0.0002$), and control and ambient samples ($p = 0.0205$). Significant differences between control and freezer samples were found at site 4 ($p = 0.0220$). Site 6 expressed a significant difference between ambient and cooler samples ($p = 0.0287$). Annual mean NH_4 concentrations (excluding spike samples

Table 2 Mean annual nitrate concentrations (mg L^{-1}) (±SE) of surface water samples

	Mean concentration (mg L^{-1}) ± SE			
	Control	Ambient	Cooler	Freezer
Site 1	0.34 ± 0.03[a]	0.33 ± 0.03[a,b]	0.51 ± 0.18[b]	0.31 ± 0.01[a,c]
Site 2	0.43 ± 0.02[a]	0.42 ± 0.07[a,b]	0.62 ± 0.18[b]	0.44 ± 0.03[a,b]
Site 3	0.12 ± 0.05[a]	0.11 ± 0.04[a,b]	0.31 ± 0.21[b]	0.13 ± 0.04[a,b]
Site 4	0.23 ± 0.12[a]	0.17 ± 0.07[a]	0.37 ± 0.29[a]	0.18 ± 0.09[a]
Site 5	0.80 ± 0.26[a]	0.85 ± 0.33[a]	0.95 ± 0.23[a]	0.75 ± 0.20[a]
Site 6	0.91 ± 0.08[a]	1.04 ± 0.12[b]	1.07 ± 0.20[a,b]	1.03 ± 0.09[a,b]
Site 7*	7.57 ± 2.23[a]	8.27 ± 2.60[a,b]	10.4 ± 3.35[b]	6.29 ± 1.75[b]
Site 8*	7.46 ± 2.27[a]	7.92 ± 2.52[a,b,c]	11.4 ± 3.58[b]	5.93 ± 1.64[a,c]

Values in rows not connected by the same letter are significantly different from each other based on ANOVA and Tukey's HSD tests (n = 14)

Control: analyzed within 3 h of collection

Ambient: held 24 h at 23°C

Cooler: held 7 days at 4°C

Freezer: held 7 days at −20°C

* Sites 7 and 8 were deionized laboratory water spiked with nutrients

7 and 8) ranged from 0.003–0.025, 0.002–0.016, 0.001–0.025, to 0.001–0.023 mg L^{-1}, in control, ambient, cooler, and freezer samples, respectively (Table 4). Kotlash and Chessman (1998) reported that where NH_4 concentrations were >0.1 mg L^{-1} samples left unpreserved for 6 days were similar to those frozen, iced, acidified, or refrigerated. If samples with low (<0.1 mg L^{-1}) NH_4 concentrations were left unpreserved for 6 days, up to 90 % of the NH_4 was lost (Kotlash and Chessman 1998).

For annual mean FOP concentrations, significant differences existed at sites 1, 2, 3, 4, and 6. Significant differences were consistently noted between the control and cooler, control and freezer, and control and ambient samples at each of the five listed sites. Excluding spiked samples 7 and 8, mean annual FOP concentrations ranged from 0.04–0.24, 0.02–0.18, 0.01–0.19, to 0.01–0.17 mg L^{-1}, respectively, for control, ambient, cooler, and freezer samples (Table 5).

No significant differences in annual mean TOP concentrations existed within sample sites. Annual mean TOP concentrations (excluding spike samples 7 and 8) ranged from 0.06–0.38, 0.05–0.36, 0.05–0.38, to 0.07–0.42 mg L^{-1}, for control, ambient, cooler, and freezer samples, respectively (Table 6).

Storage and preservation of water samples for the purpose of nitrogen and phosphorus analyses pose challenges based on biological, chemical, and physical properties of the water. No single preservation or storage method works best for all species of nutrients. Even the one concept that practically all researchers agree on – that sample filtration should take place quickly after sample collection – has its caveats, since high pressure filtration may result in cell lysis thereby altering nutrient concentrations (Gardolinski et al. 2001). Reliable nutrient data are critical in assessing many of the water quality challenges facing our Nation and the world. Researchers must continue to utilize the best storage and analyses methods available, while those developing regulations should be accommodating of limitations involved in collection and analyses of field data.

Table 3 Mean annual nitrite concentrations (mg L^{-1}) (±SE) of surface water samples

	Mean concentration (mg L^{-1}) ± SE			
	Control	Ambient	Cooler	Freezer
Site 1	0.008 ± 0.002[a]	0.005 ± 0.002[a]	0.010 ± 0.001[a]	0.008 ± 0.001[a]
Site 2	0.007 ± 0.003[a]	0.007 ± 0.003[a]	0.009 ± 0.001[a]	0.009 ± 0.002[a]
Site 3	0.004 ± 0.001[a]	0.002 ± 0.001[a]	0.005 ± 0.001[a]	0.004 ± 0.001[a]
Site 4	0.003 ± 0.001[a]	0.003 ± 0.001[a]	0.005 ± 0.001[a]	0.004 ± 0.001[a]
Site 5	0.006 ± 0.001[a]	0.009 ± 0.004[a]	0.012 ± 0.004[a]	0.009 ± 0.002[a]
Site 6	0.004 ± 0.001[a]	0.005 ± 0.002[a,b]	0.008 ± 0.001[b]	0.008 ± 0.002[a,b]
Site 7*	2.18 ± 0.42[a]	2.08 ± 0.46[a,b]	2.09 ± 0.39[a,b]	1.04 ± 0.07[b]
Site 8*	2.13 ± 0.42[a]	2.24 ± 0.44[a,c]	2.09 ± 0.39[a,c]	1.02 ± 0.05[b]

Values in rows not connected by the same letter are significantly different from each other based on ANOVA and Tukey's HSD tests (n = 14)

Control: analyzed within 3 h of collection

Ambient: held 24 h at 23°C

Cooler: held 7 days at 4°C

Freezer: held 7 days at −20°C

* Sites 7 and 8 were deionized laboratory water spiked with nutrients

Table 4 Mean annual ammonium concentrations (mg L^{-1}) (±SE) of surface water samples

	Mean concentration (mg L^{-1}) ± SE			
	Control	Ambient	Cooler	Freezer
Site 1	0.025 ± 0.008^{a}	0.016 ± 0.007^{a}	0.025 ± 0.011^{a}	0.023 ± 0.013^{a}
Site 2	0.018 ± 0.004^{a}	$0.015 \pm 0.003^{a,b}$	$0.015 \pm 0.003^{a,b}$	0.014 ± 0.005^{b}
Site 3	0.004 ± 0.003^{a}	0.002 ± 0.001^{b}	0.001 ± 0.001^{b}	0.001 ± 0.001^{b}
Site 4	0.004 ± 0.001^{a}	$0.003 \pm 0.002^{a,b}$	$0.003 \pm 0.001^{a,b}$	0.002 ± 0.001^{b}
Site 5	0.006 ± 0.005^{a}	0.009 ± 0.006^{a}	0.006 ± 0.005^{a}	0.005 ± 0.004^{a}
Site 6	$0.003 \pm 0.002^{a,b}$	0.004 ± 0.002^{a}	0.001 ± 0.001^{b}	$0.002 \pm 0.001^{a,b}$
Site 7*	0.214 ± 0.040^{a}	0.232 ± 0.043^{a}	0.209 ± 0.044^{a}	0.202 ± 0.039^{a}
Site 8*	0.223 ± 0.038^{a}	0.223 ± 0.043^{a}	0.218 ± 0.050^{a}	0.199 ± 0.040^{a}

Values in rows not connected by the same letter are significantly different from each other based on ANOVA and Tukey's HSD tests (n = 14)

Control: analyzed within 3 h of collection

Ambient: held 24 h at 23°C

Cooler: held 7 days at 4°C

Freezer: held 7 days at −20°C

* Sites 7 and 8 were deionized laboratory water spiked with nutrients

Table 5 Mean annual filtered orthophosphorus concentrations (mg L^{-1}) (±SE) of surface water samples

	Mean concentration (mg L^{-1}) ± SE			
	Control	Ambient	Cooler	Freezer
Site 1	0.12 ± 0.05^{a}	0.07 ± 0.02^{b}	0.06 ± 0.02^{b}	0.07 ± 0.01^{b}
Site 2	0.10 ± 0.06^{a}	0.04 ± 0.02^{b}	0.04 ± 0.01^{b}	0.06 ± 0.03^{b}
Site 3	0.07 ± 0.04^{a}	0.03 ± 0.02^{b}	0.02 ± 0.01^{b}	0.01 ± 0.01^{b}
Site 4	0.07 ± 0.03^{a}	0.02 ± 0.01^{b}	0.01 ± 0.01^{b}	0.02 ± 0.01^{b}
Site 5	0.24 ± 0.13^{a}	0.18 ± 0.12^{a}	0.19 ± 0.12^{a}	0.17 ± 0.11^{a}
Site 6	0.04 ± 0.01^{a}	0.02 ± 0.01^{b}	0.02 ± 0.01^{b}	0.01 ± 0.00^{b}
Site 7*	4.59 ± 0.71^{a}	4.46 ± 0.60^{a}	4.70 ± 0.61^{a}	4.25 ± 0.46^{a}
Site 8*	4.46 ± 0.60^{a}	4.53 ± 0.61^{a}	4.64 ± 0.58^{a}	4.40 ± 0.45^{a}

Values in rows not connected by the same letter are significantly different from each other based on ANOVA and Tukey's HSD tests (n = 14)

Control: analyzed within 3 h of collection

Ambient: held 24 h at 23°C

Cooler: held 7 days at 4°C

Freezer: held 7 days at −20°C

* Sites 7 and 8 were deionized laboratory water spiked with nutrients

Table 6 Mean annual total orthophosphorus concentrations (mg L^{-1}) (±SE) of surface water samples

	Mean concentration (mg L^{-1}) ± SE			
	Control	Ambient	Cooler	Freezer
Site 1	0.29 ± 0.06^{a}	0.29 ± 0.04^{a}	0.31 ± 0.06^{a}	0.42 ± 0.16^{a}
Site 2	0.18 ± 0.05^{a}	0.17 ± 0.04^{a}	0.20 ± 0.03^{a}	0.23 ± 0.11^{a}
Site 3	0.06 ± 0.02^{a}	0.06 ± 0.02^{a}	0.05 ± 0.01^{a}	0.09 ± 0.02^{a}
Site 4	0.11 ± 0.03^{a}	0.05 ± 0.01^{a}	0.08 ± 0.02^{a}	0.10 ± 0.02^{a}
Site 5	0.38 ± 0.13^{a}	0.36 ± 0.14^{a}	0.38 ± 0.14^{a}	0.37 ± 0.12^{a}
Site 6	0.22 ± 0.13^{a}	0.06 ± 0.01^{a}	0.05 ± 0.02^{a}	0.07 ± 0.02^{a}
Site 7*	4.28 ± 0.67^{a}	4.02 ± 0.48^{a}	4.24 ± 0.46^{a}	4.16 ± 0.44^{a}
Site 8*	4.39 ± 0.62^{a}	4.05 ± 0.50^{a}	4.56 ± 0.59^{a}	4.16 ± 0.43^{a}

Values in rows not connected by the same letter are significantly different from each other based on ANOVA and Tukey's HSD tests

Control: analyzed within 3 h of collection

Ambient: held 24 h at 23°C

Cooler: held 7 days at 4°C

Freezer: held 7 days at −20°C

* Sites 7 and 8 were deionized laboratory water spiked with nutrients

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