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Isotopic Differentiation ($\delta^{18}O_{PO4}$) of Inorganic Phosphorus among Organic Wastes for Nutrient Runoff Tracing Studies: A Summary of the Literature with Refinement of Livestock Estimates for Grand Lake St. Marys Watershed (Ohio)

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Abstract: The use of stable isotopes, specifically $\delta^{18}O_{PO4}$ ratios, in differentiating potential sources of inorganic phosphorus (e.g., wastewater, septic, wild animals, domesticated animals, livestock, substrates, or commercial fertilizers) to watersheds is a growing field. This method produces data that, used in conjunction with statistical mixing models, enables a better understanding of contributing sources of runoff. However, given the recent development of this research area there are obvious limitations that have arisen, due in large part to the limited available reference data to compare water samples. Here, we attempt to expand the availability of reference samples by applying stable isotope methods to three types of common agricultural manures: poultry, dairy, and swine. We also aim to concatenate the organic waste literature on this topic, creating a more robust comparison database for future study and application in phosphorus source partitioning research. Among our samples, $\delta^{18}O_{PO4}$ ratios for poultry were considerably elevated compared to dairy and swine manures (values of 18.5‰, 16.5‰, and 17.9‰, respectively). Extending this to other published ratios of $\delta^{18}O_{PO4}$ from various types of waste products (e.g., septic, wastewater, livestock, other animals), a total range from 8.7‰ to 23.1‰ emerged (with existing poultry manure samples also ranking among the highest overall). Variation among samples in the larger dataset demonstrates the need for a further compilation of $\delta^{18}O_{PO4}$ ratios for various types of waste, especially specific to geographic regions and watershed scales. With an increased sample size, the statistical strength associated with these methods would greatly improve.

Keywords: algal blooms; nutrient sources; oxygen isotopes; phosphates

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1. Introduction

Harmful algal blooms (HABs) are an emerging environmental concern that has gradually experienced an increase in frequency and severity in the Great Lakes and Ohio River basins over the past several decades [1]. Through toxin production and sheer biovolume, HABs negatively affect the availability of drinking water, recreation potential, ecosystem integrity, economies, human and animal health, and more [2]. In the Midwestern United States, this often takes the form of microcystin toxins from a small handful of near-monoculture blue-green algal taxa [3]. Catalyzing harmful algal blooms is nutrient runoff, which primarily results from diffuse nonpoint sources from agriculture-rich regions [4]. Numerous efforts are ongoing in the Great Lakes and Ohio River watersheds and beyond to mitigate and reduce nutrient loading through a variety of on-field best management practices (BMPs) as well as habitat restoration projects; however, these efforts still need to be scaled up to further improve water quality in line with most conservation benchmarks [5–7].

Specific to conservation implementation, one of the most challenging aspects is the placement of practices. The identification of critical pollution source areas is key to maximizing effect, as not every acre in a given watershed contributes to nonpoint source runoff

equally [8]. Some best management practices are obviously generalized (e.g., cover crops, manure management), while others are more targeted to particular areas (e.g., phosphorus filters, bioreactors, saturated buffers), which presents challenges in determining where to place and how to scale up on a watershed level [9]. Understanding sources of nutrients, which can include wastewater effluent, septic tanks, animal manure, internal loading from lake or river sediments, inorganic fertilizer products, etc., and what fraction of surface water loading they ultimately contribute to can greatly inform watershed management strategies. Thus, there is a need for definitive methods of identifying phosphorus sources.

Isotopic methods for tracing problematic nutrients such as phosphorus entering aquatic systems are gaining traction in this field. Simply, these methods compare ratios of heavy to light isotopic atoms to analyze differences among specific samples. Unfortunately, phosphorus has only one stable isotope, ^{31}P, making it impossible for direct use in distinguishing sources. However, phosphorus, in nature, is typically tightly bound to oxygen atoms in the form of phosphate (PO_4), making it possible to analyze the oxygen isotope ratios within PO_4. This method has been refined and utilized within multiple watersheds throughout the world [10–13]. One such study determined the phosphate contributions of each waterway entering San Francisco Bay [10], while another analyzed $\delta^{18}O_{PO4}$ ratios of mining and phosphate rock runoff contributing to the Huangbai River, China [13]. As a result of these and other studies, data regarding more specific P sources is starting to accumulate. For example, some ratios have been established for wastewater effluent [11,14], individual septic tanks [14,15], synthetic fertilizers [11,12], and a few animal wastes [11,16]. However, despite the uptick in this literature, there is still limited information on the $\delta^{18}O_{PO4}$ ratios for varying types of animal wastes, particularly in the Midwestern United States. Considering that agricultural runoff is a large contributor of nutrients to aquatic systems, more data on specific manures is necessary.

We aim to contribute to this growing field through an analysis of various manures from agricultural practices in Northwest Ohio and broaden the field of reference for isotopic ratio methods. Here, we combine the limited existing data regarding the $\delta^{18}O_{PO4}$ of both human and animal wastes with those of our own collection. We then compare these values in order to evaluate this method in differentiating types of wastes to help inform future mixing models that attempt to discern P sources from a conservation lens.

2. Methods

2.1. Sample Collection and Processing

Three types of agricultural manures were collected within this study: poultry, swine, and dairy. Samples were provided from a total of 7 independent farms. These manures were supplied via anonymous donations from local operations within Mercer County, Northwest Ohio (Figure 1). Due to the anonymity of the manures supplied, specific sampling techniques and locations cannot be described.

Kept frozen until processed, manure samples were diluted with distilled water and mixed well. Thirty milligrams of each type of manure were added to 2 L of distilled water and manually shaken repeatedly over the course of an hour. These mixtures were pumped through high-volume filtration apparatus to avoid isotope fractionation and remove large particulates (Grade 4 Whatman filter with 20–25 µm particle retention) (Cytiva, Marlborough, MA, USA). The filtered samples were split into subsamples to be analyzed for both $\delta^{18}O_{PO4}$ and dissolved reactive phosphorus (DRP) concentrations. DRP was quantified following the ascorbic acid method (EPA 365.1) and analyzed on a HACH DR3900 Benchtop VIS Spectrophotometer (HACH Company, Loveland, CO, USA). The filtered subsample designated for $\delta^{18}O_{PO4}$ analysis was used in the precipitation of solid silver phosphate (Ag_3PO_4). The phosphate within the filtered liquid samples was bound by magnesium-induced co-precipitation (MagIC) with the addition of magnesium chloride and sodium hydroxide [17]. This solid settled to the base of the vessel, allowing the removal of the supernatant using a peristaltic pump. After the removal of as much overlying liquid as possible, the MagIC was again dissolved in the minimum amount of 10 M Nitric Acid and

passed through OASIS HLB filtration cartridges (Waters, Milford, MA, USA) to remove any organic contaminants and remaining small particulates. The product was then processed through a series of dissolutions and precipitations via methods detailed by McLaughlin et al. 2004 [18]. Ultimately, solid Ag_3PO_4 samples were packaged and sent in triplicate to the Yale Analytical and Stable Isotope Center (YASIC) for analysis using a TC/EA (thermochemical elemental analyzer) along with a Thermo DeltaPlus XP Isotope Ratio Mass Spectrometer (IRMS) (Thermo Fisher Scientific, Waltham, MA, USA).

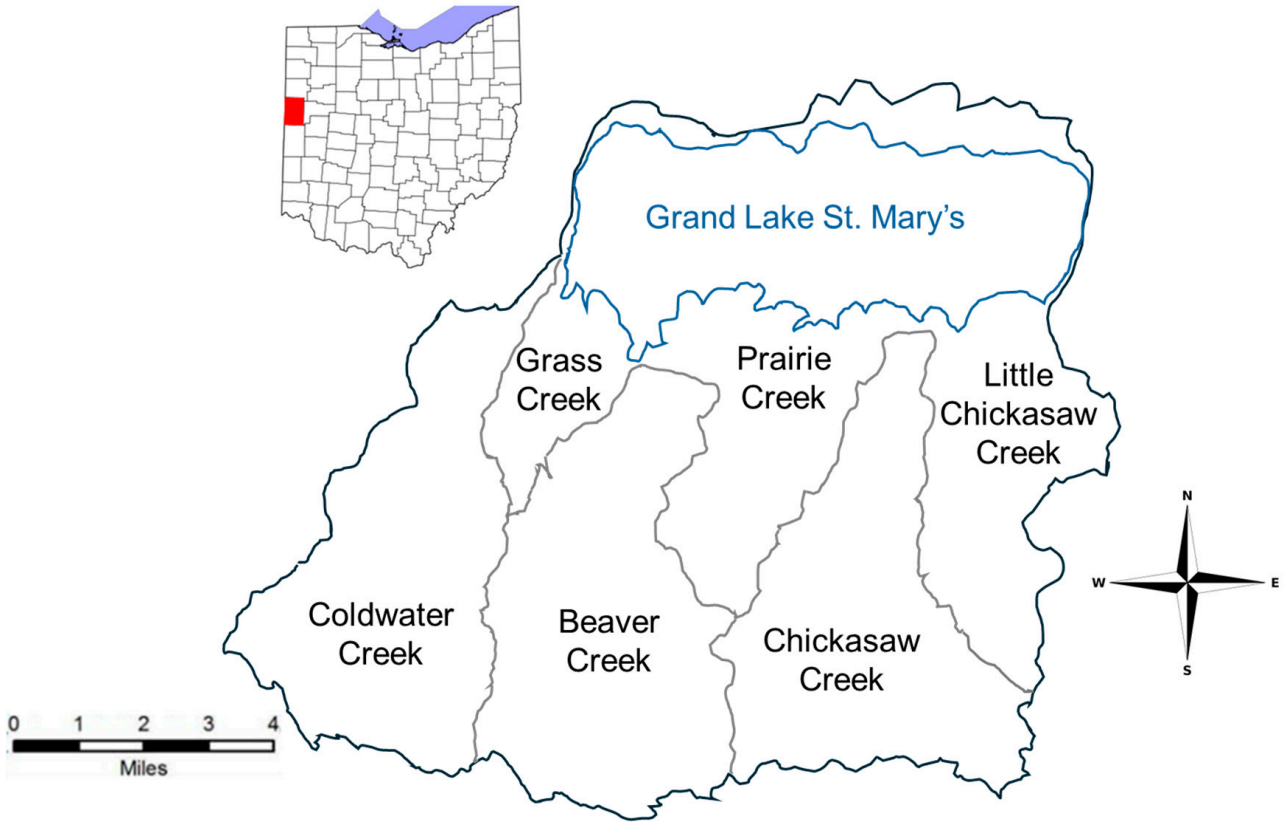


Figure 1. Map of the Grand Lake St. Mary's watershed. Subwatersheds are outlined in grey and the lake itself is outlined in blue. The red inlay illustrates the location of Mercer County within Northwest Ohio. Samples collected for this study were collected anonymously within this region.

2.2. Data Analysis

Data analysis within this study incorporated both $\delta^{18}O_{PO4}$ ratios resulting from previously described collected manure samples as well as $\delta^{18}O_{PO4}$ ratios found within similar studies and the existing literature (see Supplementary Information for raw data). Differences among types of waste were assessed using a One-Way ANOVA with an associated Tukey's post hoc analysis when appropriate. This analysis only included waste types that are relevant to freshwater systems and have a sample size greater than n = 1. Given the relatively low sample sizes often inherent in these kinds of studies, differences were considered statistically significant at $\alpha = 0.1$. Statistical analyses were conducted using R software version 4.1.2 [19].

3. Results

3.1. Collected Wastes

Inorganic phosphorus concentrations for the manure samples collected within this study ranged from 1.42–33.35 mg/L. These values are available in the Supplementary Materials (Table S1). An analysis of $\delta^{18}O_{PO4}$ ratios from poultry (n = 3), swine (n = 2), and dairy (n = 2) manures collected within this study revealed values ranging from 16.2–19.1‰ (Figure 2). Ratios among poultry samples (mean = 18.5‰) and swine samples (mean = 17.9‰)

were relatively similar, but we did find dairy samples (mean = 16.5‰) to have significantly lower ratios than that of poultry (df = 2, $p = 0.06$) (Tukey's poultry-swine $p = 0.61$, swine-dairy $p = 0.17$, poultry-dairy $p = 0.05$). These $\delta^{18}O_{PO4}$ ratios, as well as the %O recovered from each sample, are detailed within the Supplementary Information (Table S2).

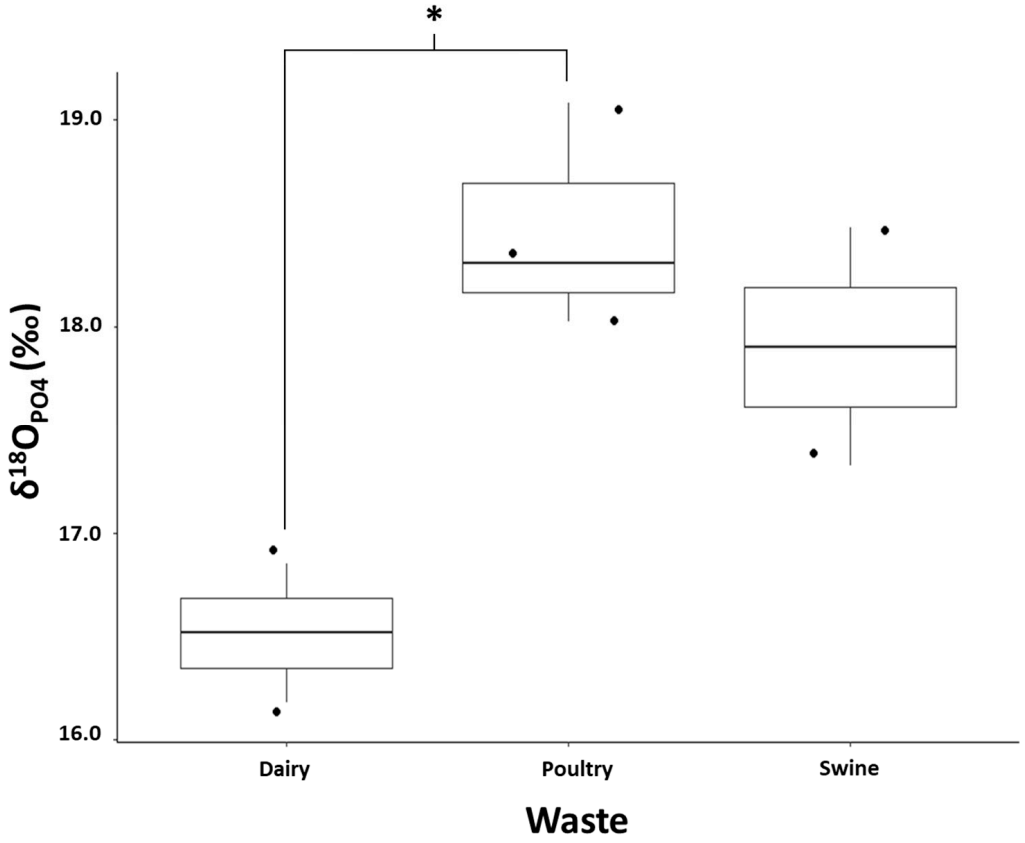


Figure 2. Boxplot of $\delta^{18}O_{PO4}$ ratios of waste types collected within this study. Poultry manure samples were significantly elevated relative to dairy manure samples. The large asterisk represents significant differences among samples ($\alpha = 0.1$).

3.2. Established Values

Collection of existing data regarding the $\delta^{18}O_{PO4}$ ratios of various types of human and animal wastes produced the following categories: treated wastewater effluent (n = 46), individual septic systems (n = 4), dairy manure (n = 4), swine manure (n = 5), poultry manure (n = 6), seabird guano (n = 10), dog waste (n = 1), and Canada Goose waste (n = 1) (Table S3) (Figure 3). These ratios ranged from 8.7‰ to 23.1‰ (Figure 3). Our compilation of data suggests that the range of ratios for wastes relevant to freshwater systems (and with n > 1) is slightly more restricted at 8.7‰ to 19.7‰ (Figure 4). Overall, we found considerable overlap among values, as wastewater effluent alone encompassed the entire range of waste types here. However, an analysis of freshwater-relevant ratios (excluding dog, goose, and seabird wastes) did reveal poultry waste to be highest and significantly elevated in comparison to wastewater effluent results (df = 4, $p = 0.04$) (Tukey's wastewater-poultry $p = 0.07$) suggesting that some level of data partitioning and application is plausible.

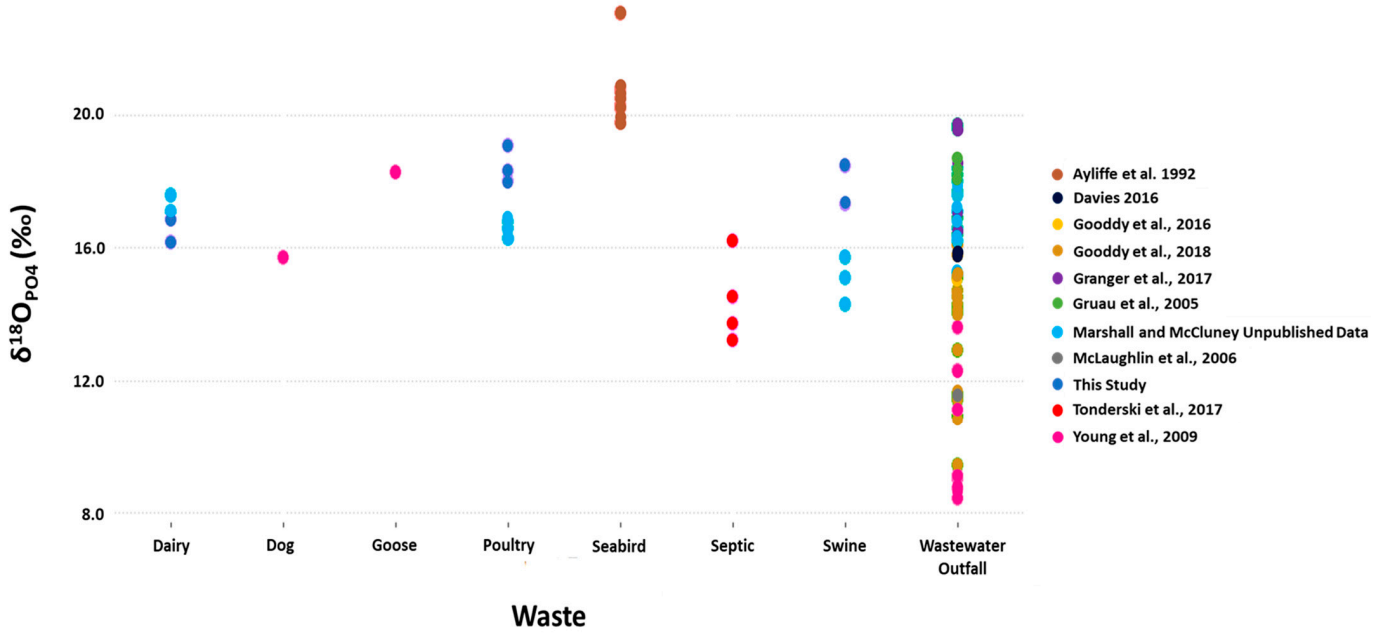


Figure 3. $\delta^{18}O_{PO4}$ ratios of all waste samples found within the literature as well as those analyzed within this study [10,11,14–16,20–23]. Sources for each data point are indicated according to color.

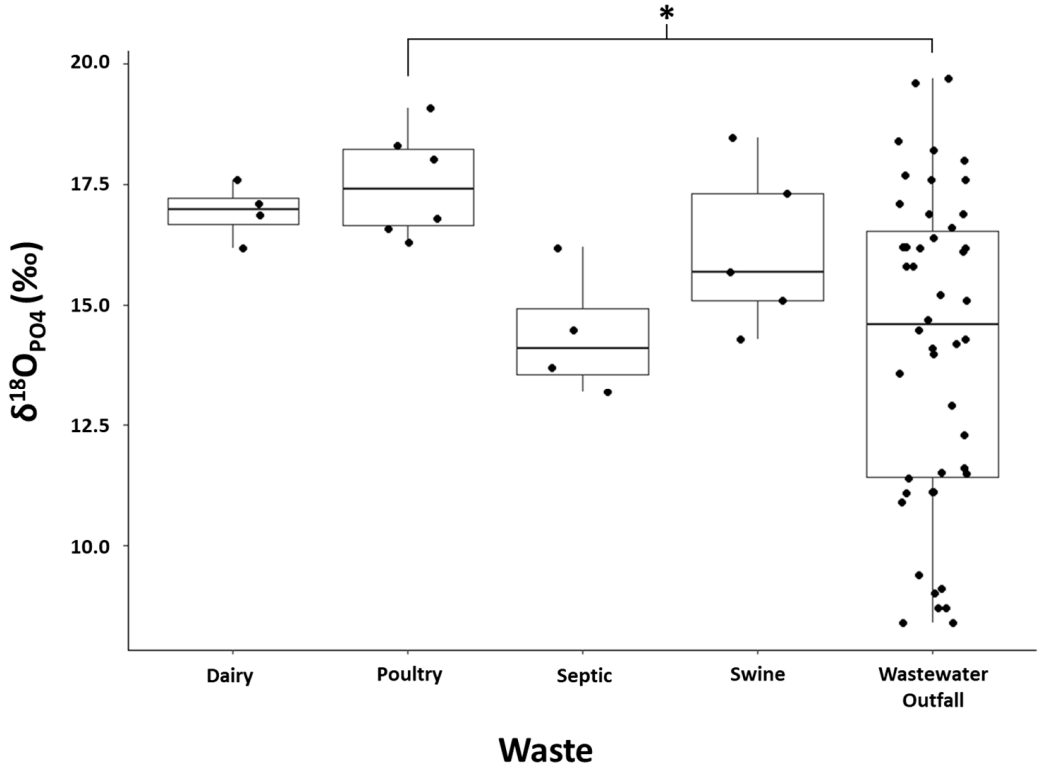


Figure 4. $\delta^{18}O_{PO4}$ ratios of data used for One-Way ANOVA analysis for differences among types of manure. All samples were included if relevant to freshwater systems and included a sample size greater than 1 (n > 1). Most values fell within similar ranges with significant elevation found comparing poultry samples and those from wastewater outfall locations. The large asterisk represents significant differences among samples ($\alpha = 0.1$).

4. Discussion

The use of $\delta^{18}O_{PO4}$ ratios is a growing field that shows promise. If refined, the values produced via this method could be used to determine the relative contributions of inorganic

phosphates by specific sources. This will allow for concentrated efforts to prevent nutrient losses in watersheds and better allocation of funds and resources. The specific values found in this study, as well as the others summarized herein, would be most valuable in the form of a database that others could pull from in distinguishing P sources. For example, a recent study by Wang et al. 2023 used mixed-end member and Bayesian modeling to determine the impacts of several inorganic phosphorus sources within the Yangtze River Catchment in China by comparing isotopic values from the waterway alongside reference values [24]. Similarly, in the Yasu River Watershed in Japan, researchers utilized multiple regression analysis, examined linear multicollinearity, and employed Akaike's Information Criterion (AIC) to explore the inputs of nutrients from various bedrock and land use categories [12]. These methods, incorporating baseline nutrient isotope data into statistical mixing models, have also been applied successfully in North America within Lake Erie [11] and San Francisco Bay Watersheds [10].

While these methods have been successfully applied in sourcing studies, the use of $\delta^{18}O_{PO4}$ ratios of potential sources of both point and non-point nutrients still presents challenges. Here, we highlight these challenges specific to data on human and animal wastes, as the existing literature regarding these values is highly limited by sample size and geographic range. Including our addition of data, we found and analyzed a total of 77 ratios, with some having sample sizes as low as n = 1. This alone provides evidence for increased sample frequency and abundance to be able to better apply statistical analyses with the strength to support using isotopic methods as a more global solution in identifying primary P contributors. Additionally, our study also found notable amounts of overlap among $\delta^{18}O_{PO4}$ ratios (Figures 2–4) that should be discussed and better understood.

Possible explanations for the variability and overlap in isotope ratios across the dataset may come from sampling techniques. Processing at wastewater treatment facilities can be specific at the watershed or regional scale, causing fluctuation in $\delta^{18}O_{PO4}$ ratios in final effluent samples [14]. In addition, agricultural practices can also be unique to location due to species of livestock and managerial practices. Of the three species from which manure samples were obtained for this study, two are classified as monogastrics (poultry and swine) and one is classified as a ruminant (dairy cattle). Among these species, we see various differences based on their physiology, which translates to a variation in nutrients found in their manure. Additionally, the diets provided at specific livestock operations are expected to vary as the included ingredients fluctuate by availability, cost, and time of year. Growing season, soil quality, management and harvest practices, and storage methods, which are also somewhat dictated by region, impact the nutritional profile and digestibility of feed ingredients, thus having a potential impact on isotopic variation. Lastly, a lack of uniform collection methods for waste samples in isotope analysis is also an important aspect. For instance, the collection of wastewater effluents in existing studies has been executed at various points within treatment facilities, including outfall locations [14], versus treated effluent structures within the facility [12]. It can also prove difficult to representatively collect agricultural samples [25], with dry matter content, collection tools, and location within operations as factors that may need to be standardized. Relating to this, anonymity among suppliers of agricultural manures may also be a sensitive topic and must be addressed according to the study. Moving forward, a more uniform collection standard should be implemented.

5. Conclusions

This work contributes towards narrowing the deficit of $\delta^{18}O_{PO4}$ ratios available for human and animal wastes while also exemplifying the need for further work in the field, as substantial gaps in the literature are apparent. With the intention of providing additional data from our own study area, we suggest a library of reference samples be established for future use. In this reference library, additional isotopic sources from various waste types spanning a wider geographic region are going to be needed if a representative series of index values is to be built. Having this library of index samples is quite nearly a prerequisite

to interpreting data from lakes and streams. With the development of a larger library style dataset, there exists the potential to greatly contribute to improving sourcing efforts of phosphorus runoff, providing valuable information towards mitigating the driving factors that underpin HABs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pollutants4030021/s1, Table S1: Inorganic Phosphorus Concentrations; Table S2: $\delta^{18}O_{PO4}$ ratios from this study only; Table S3: $\delta 18OP_{O4}$ ratios and n values for all waste samples.

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