Stable isotopic composition of perchlorate and nitrate accumulated in plants: hydroponic experiments and field data

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Abstract

Natural perchlorate (ClO_4^-) in soil and groundwater exhibits a wide range in stable isotopic compositions ($\delta^{37}\text{Cl}$, $\delta^{18}\text{O}$, and $\Delta^{17}\text{O}$), indicating that ClO_4^- may be formed through more than one pathway and/or undergoes post-depositional isotopic alteration. Plants are known to accumulate ClO_4^- , but little is known about their ability to alter its isotopic composition. We examined the potential for plants to alter the isotopic composition of ClO_4^- in hydroponic and field experiments conducted with snap beans (*Phaseolus vulgaris* L.). In hydroponic studies, anion ratios indicated that ClO_4^- was transported from solutions into plants similarly to NO_3^- but preferentially to Cl^- (~ 4X). The ClO_4^- isotopic compositions of initial ClO_4^- reagents, final growth solutions, and aqueous extracts from plant tissues were essentially indistinguishable, indicating no significant isotope effects during ClO_4^- uptake or accumulation. The ClO_4^- isotopic

composition of field-grown snap beans was also consistent with that of ClO_4^- in varying proportions from irrigation water and precipitation. NO_3^- uptake had little or no effect on NO_3^- isotopic compositions in hydroponic solutions. However, a large fractionation effect with an apparent ϵ ($^{15}\text{N}/^{18}\text{O}$) ratio of 1.05 was observed between NO_3^- in hydroponic solutions and leaf extracts, consistent with partial NO_3^- reduction during assimilation within plant tissue. We also explored the feasibility of evaluating sources of ClO_4^- in commercial produce, as illustrated by spinach, for which the ClO_4^- isotopic composition was similar to that of indigenous natural ClO_4^- . Our results indicate that some types of plants can accumulate and (presumably) release ClO_4^- to soil and groundwater without altering its isotopic characteristics. Concentrations and isotopic compositions of ClO_4^- and NO_3^- in plants may be useful for determining sources of fertilizers and sources of ClO_4^- in their growth environments and consequently in food supplies.

Keywords

Oxygen isotopes; Chlorine isotopes; Nitrogen isotopes; Plant uptake; Isotope fractionation in snap beans (*Phaseolus vulgaris* L.); Irrigation

1 Introduction

Perchlorate (ClO_4^-) is a ubiquitous trace inorganic constituent in the environment with both natural and anthropogenic sources. Widespread occurrence of natural ClO_4^- has been documented and there are numerous anthropogenic sources of ClO_4^- (Rajagopalan et al., 2009; Rao et al., 2007; Kounaves et al., 2010; Böhlke et al., 2009; Jackson et al., 2015). Because of its potential impact on thyroid function, the U.S. Environmental Protection Agency (EPA) is currently in the process of establishing a maximum contaminant level (MCL) for ClO_4^- in drinking water (Greer et al., 2002; “Drinking Water,” 2011). Leafy edible plants (e.g. lettuce and spinach), fruits and vegetables, and forage species are also an exposure route for humans, either from direct ingestion or when used for dairy feed that can lead to elevated concentrations of

ClO₄⁻ in human breast milk and animal milk (Dasgupta et al., 2006; Jackson et al., 2005; Kirk et al., 2003; 2007; Sanchez et al., 2005).

Differentiating between various sources of ClO₄⁻ is possible based on the stable isotopic composition of ClO₄⁻. The Cl and O stable isotopic composition of ClO₄⁻ has been shown to vary among samples representing different major sources or geographic areas (Jackson et al., 2010; Sturchio et al., 2011). Natural ClO₄⁻ has variable but generally positive $\Delta^{17}\text{O}$ values reflecting formation reactions involving ozone (O₃) and is thought to be derived largely from atmospheric deposition (Jackson et al., 2010; Sturchio et al., 2011). However, isotopic variation of natural ClO₄⁻ indicates that multiple production mechanisms may exist, or that post-depositional alteration (fractionation and/or exchange) can alter the original isotopic composition. Processes that could alter ClO₄⁻ isotopic compositions in the environment include microbial transformation, diffusion, isotope exchange with water, and plant uptake/transformation (Jackson et al., 2010; Van Aken and Schnoor, 2002; Sturchio et al., 2007; Hatzinger et al., 2009).

Microbial degradation produces a well-described mass-dependent effect on $\delta^{37}\text{Cl}$ and $\delta^{18}\text{O}$, but degradation alone cannot explain the isotopic variation among different geographic locations (Sturchio et al., 2007; Hatzinger et al., 2009; Sturchio et al., 2003). Isotopic fractionation of ClO₄⁻ by transport-related processes (dissolution/precipitation and diffusion) has not been thoroughly investigated, but such effects are likely to be smaller than the observed isotopic variations of natural ClO₄⁻ (Desaulniers et al., 1986; Kaufmann et al., 1993; Ransom et al., 1995). Isotope exchange of O between ClO₄⁻ and coexisting substances, such as water, could affect ClO₄⁻ isotopic composition, but experiments indicate the ClO₄⁻ - H₂O exchange half-life is greater than 100 years in the absence of catalysts (Böhlke et al. 2005; Hoering et al., 1958).

The effect of plant uptake on ClO_4^- isotopic compositions is not yet clear. Numerous soil and hydroponic exposure experiments (Sanchez et al., 2005; Seyfferth and Parker, 2007; Seyfferth et al., 2008 ab; Ha et al., 2011; Voogt and Jackson, 2010) have indicated the potential for plants to uptake and accumulate ClO_4^- . It is possible that plants could change the ClO_4^- isotopic composition through a variety of mechanisms including: transport carriers in the root; diffusion limitations through the root; reduction of ClO_4^- within the plant; translocation within the plant; and exchange of O between ClO_4^- and H_2O or other compounds catalyzed by plant enzymes. It is well-known that physiological and physicochemical processes (fixation, assimilation, and diffusion) in plants can cause isotopic fractionation of carbon (Marshall et al., 2007; Farquhar et al., 1989; Schmidt et al., 2015) ($^{13}\text{C}/^{12}\text{C}$) and nitrogen ($^{15}\text{N}/^{14}\text{N}$) (Schmidt et al., 2015; Evans, 2001; Mariotti et al., 1981; Werner and Schmidt, 2002); likewise, intracellular processing of ClO_4^- within plants may also affect ClO_4^- isotopic compositions. There are conflicting published results concerning ClO_4^- reduction in plants (Van Aken and Schnoor, 2002; Seyfferth et al., 2008b). Understanding whether or not plants can alter the isotopic composition of ClO_4^- is important because such alteration could introduce complications for determining sources of ClO_4^- in plants, soils, and groundwaters.

In the current study, controlled experiments were conducted to develop procedures for analyzing the isotopic composition of ClO_4^- extracted from plants and to determine whether isotopic exchange or fractionation (by transport or degradation) might alter the isotopic composition of ClO_4^- accumulating in plants. This was accomplished by comparing the stable isotopic compositions of ClO_4^- accumulated in hydroponically grown snap bean plants (*Phaseolus vulgaris* L.) with those of the starting reference materials and growth solutions. Results were interpreted in part by contrasting the isotopic fractionation of ClO_4^- and NO_3^- in the

same experiments. This is important because isotopic fractionation effects of enzymatic reactions may not fully be expressed in residual reactants (i.e. in heterogeneous systems where reactions are transport-limited). Therefore, a lack of observable isotope effects in extractable ClO_4^- might not be a reliable indicator of ClO_4^- stability unless extractable NO_3^- exhibited substantial fractionation effects. We also evaluated ClO_4^- isotopic composition in plants and source water for field-grown snap beans to validate the applicability of our methods in a more realistic setting. In order to demonstrate how our methods can be used to identify sources of ClO_4^- in the food chain, we analyzed commercially grown spinach and snap bean plants exposed only to background concentrations of ClO_4^- .

2 Materials and Methods

2.1 Hydroponic Snap Bean Studies

Hydroponic greenhouse studies were conducted at the Texas Tech University Greenhouse Complex in Lubbock, TX. Snap beans (*Phaseolus vulgaris* L.) were grown hydroponically in a nutrient solution containing commercial ClO_4^- and/or a USGS ClO_4^- standard. Snap beans were selected for the hydroponic study as part of more comprehensive ongoing growth studies by the Agricultural Research Service (ARS) of the U.S. Department of Agriculture (USDA), which are described in the snap bean field studies section below. Details regarding the growth, collection, and analysis of the snap bean plants in the hydroponic study are described below.

Two hydroponic experiments were conducted with multiple treatments in each experiment (Table 1). Treatments generally consisted of snap beans (*Phaseolus vulgaris* L.) grown in nutrient solutions containing ClO_4^- (0.01, 2, and 10 mg/L) along with NO_3^- (235-274 mg NO_3^- -N/L). The 2 and 10 mg/L ClO_4^- treatments were used in order to reduce the amount of plant mass needed to be extracted for isotopic analysis while the 0.01 mg/L ClO_4^- treatment was used to approximate realistic values of ClO_4^- observed in nature (Rajagopalan et al., 2009; Rao et

al., 2007) and to compare the relative differences in uptake between low and high concentrations of source ClO_4^- . Two control treatments were also evaluated. One control contained no plants and 1 mg/L ClO_4^- and the other contained plants and no ClO_4^- . To minimize ambiguity in the results, two sources of ClO_4^- were used in the experiments.

The ClO_4^- for the 0.01, 1, and 10 mg/L exposures was supplied using a synthetic 98% sodium perchlorate (NaClO_4) lab reagent (ACROS Organics) with normal synthetic isotopic composition, while the ClO_4^- used in the 2 mg/L exposure was a potassium perchlorate (KClO_4) reagent (RSIL5) that had anomalously low $\delta^{37}\text{Cl}$, high $\delta^{18}\text{O}$, and large non-mass-dependent ^{17}O anomaly (Böhlke et al., 2017). These contrasting initial isotopic compositions enabled distinctions to be made between isotopic fractionation, exchange, and mixing.

Snap bean seeds were sown in small rockwool cubes presoaked in reverse osmosis (RO) water. The seeds were watered daily with RO water until full germination (presence of 4-5 leaves, ~ 3 weeks). During the germination period, the small rockwool cubes were transferred to larger rockwool cubes. Polypropylene/polyethylene opaque tubs (52.7cm × 36.87cm × 33.3cm) were pre-rinsed with RO water three times and were filled with 32 L and 34 L (for experiment 1 and experiment 2, respectively) of a standard nutrient solution (Voogt and Jackson, 2010) with minor modifications. Prior to transfer of the plants to the growth tubs, the pH of the nutrient solution was adjusted to between 5.5 and 6.1 by addition of HNO_3 . The rockwool cubes containing plants were then positioned on the lids of the tubs, with the plant roots suspended into the nutrient solution and plant shoots exposed to air. Rockwool cubes were covered with aluminum foil to prevent algae growth. Commercial aquarium pumps supplied air to maintain bulk oxic conditions in solution for the duration of the experiments.

After germination reached the 4-5 leaf stage (~15-18 days), plants were exposed to ClO_4^- by addition of a concentrated solution in selected tubs. The water level in each tub was maintained by adding RO water weekly. Growth solution samples from each tub were collected every two to three days and refrigerated immediately until analyzed. Electrical conductivity and pH were also measured two to three times weekly. pH was maintained between 5.5 and 6.1 using 3M KOH. After a few weeks, insects became apparent and leaves were treated using Safer Soap (AgroCrop Sciences) or Orthonex (an organophosphate insecticide).

Snap bean plants were harvested 62–65 days after germination, corresponding to 38 days of ClO_4^- exposure. The bean pods, leaves, stems, and roots from each treatment were separated, weighed, and frozen until processed. All treatments were evaluated to determine the amount of ClO_4^- and NO_3^- lost from solution over time and accumulated in plant tissue, while a subset of treatments (1 mg/L no plants control, 2 mg/L, and 10 mg/L ClO_4^-) were evaluated for stable isotopes.

The plant-solute extraction method of Ellington and Evans (2000) was used in these experiments with minor modifications. The total mass of leaves collected per replicate (~ 85 – 400 g) in each treatment was extracted in 20 g fresh weight (FW) portions. The bean pods, stems, and roots were separated into approximately 5, 6, and 10 g FW portions, respectively. Each portion of leaves, bean pods, stems, and roots were placed in containers (vials) with 200, 25, 25, and 20 mL of distilled deionized (DDI) water, respectively. Water/plant tissue mixtures were boiled for 30-60 minutes in a precision boiler set at 99°C. The boiled extracts were centrifuged and the supernatant was decanted into containers (vials) with activated alumina (Al_2O_3) and placed in the refrigerator overnight to remove organics. Each leaf aqueous extract was then vacuum filtered using GF/F 47 mm Whatman™ filters. The bean pods, stem, and root

extracts were filtered using syringe Whatman™ filters. From each tissue extraction solution, a small volume was separated and cleaned by passing the aqueous extract through a (0.2 µm) syringe filter and a Dionex OnGuard® RP cartridge to further remove organic substances. Cleaned samples from each treatment were analyzed for ClO_4^- , Cl^- , NO_3^- -N, SO_4^{2-} , and PO_4^{3-} , and a small volume was set aside and preserved for NO_3^- isotopic analysis by raising the pH to 11-12 using 50% w/w NaOH. The remaining filtered supernatants of all leaf extractions per replicate were pooled together to make one extraction solution per replicate treatment for ClO_4^- isotopic analysis. The pooled leaf extracts for each replicate treatment as well as the remaining nutrient solutions of each replicate treatment, excluding the 0.01 mg/L and no ClO_4^- control plant extraction and growth solutions, were pumped through Purolite® A-530E resin columns to capture ClO_4^- (Böhlke et al., 2017; Hatzinger et al., 2011). Columns were preserved with 0.1 N HCl ($\text{pH} \leq 2$) and stored at 4°C until processing.

2.2 Snap Bean Field Studies

Ozone-sensitive (S156) and ozone-tolerant (R123) snap bean genotypes described previously (Burkey et al., 2005) were grown in field plots during the summers of 2009 – 2011 at two locations to provide bulk leaf tissue for extraction and isotopic analysis of ClO_4^- . The first site was located at the USDA-ARS Air Quality field site 5 km south of Raleigh, NC (Lat: 35°43'59" N; Long: 78°41'2" W) and the second site was located at the Long Island Horticultural Research & Extension Center, Riverhead, NY (Lat: 40°57'36" N; Long: 72°43'12" W). For each location in each year, three separate plots consisting of 500 linear feet of row were established as three experimental blocks with each block consisting of one S156 plot and one R123 plot (randomized complete block design). Fields were cultivated before planting and a '10-10-10 Extra' fertilizer blend (Carolina Eastern-Vail, Niverville, NY), with an average ClO_4^-

concentration of less than 45 $\mu\text{g/kg}$ -dry weight, was applied at a rate of 500 lbs/acre (0.056 kg/m^2) by broadcast and incorporated into the soil prior to planting. Approximately 1500 seeds were sown per plot in late May or early June each year at both sites. Plots were provided with water equivalent to at least 1 inch of rain per week by either natural rainfall or supplemental drip irrigation from wells located at each site. In preliminary tests, well water at the Raleigh, NC site was found to contain concentrations of ClO_4^- up to 5 $\mu\text{g/L}$ in excess of typical background concentrations ($< 0.005 - 1 \mu\text{g/L}$) (Rajagopalan et al., 2009; Rao et al., 2007). In this case, a Purolite® A-530E ion exchange resin column was used to reduce ClO_4^- concentration for irrigation to within typical background values. Irrigation water at the NY site was found to contain $\sim 0.3 \mu\text{g/L}$ ClO_4^- so no resin column was used. At 45-55 days after planting at the developmental stage, when vegetative biomass was near maximum, leaf tissue was manually harvested from each plot, placed in mesh bags, and dried at 60 °C. Dried leaf tissue was stored in large plastic bags to exclude moisture and prevent contamination and then shipped to Texas Tech University for ClO_4^- extraction and purification.

Field grown snap bean leaves were extracted in bulk two times for 48 hours in large 55-gallon barrels using reverse osmosis water to which the pH was reduced to 2 using HCl. Leaves were removed from solution and the solution was pumped through a 0.45 μm filter and Purolite® A-530E resin columns. The resin columns were stored at 4°C until processed for ClO_4^- isotopes.

2.3 Commercial Spinach Sample

Fresh spinach leaves (38 kg) in pre-packaged plastic bags were purchased from a supermarket in Lubbock, TX in early November, 2012. Spinach sold by the supplier was conventionally grown by contract farms in the Salinas Valley, CA region from April through

October and the Yuma, AZ region from November through March. No other information about growth conditions for this product are available.

Perchlorate was extracted from spinach using the same extraction procedure as the field grown snap bean leaves. Leaves were removed from solutions and the extraction solution was pumped through a 0.45 µm filter and Purolite® A-530E resin column. The extraction column was stored at 4°C until processed for ClO₄⁻ isotopes.

2.4 Anion Concentration Measurements

Concentrations of ClO₄⁻ and major anions (Cl⁻, NO₃⁻, SO₄²⁻, PO₄³⁻) in hydroponic solutions and plant extracts were measured at Texas Tech University using sequential ion chromatography-mass spectroscopy-mass spectroscopy (IC-MS/MS) with a reporting limit of 0.05 µg/L (ClO₄⁻) and ion chromatography (EPA Method 300.0) with a reporting limit of 0.5 mg/L (other anions) as described in Rao et al. (2007). To facilitate comparisons of measured concentrations, plant bioconcentration factors (BCF) were calculated according to Equation 1 (Newman and Clements, 2008).

$$BCF\left(\frac{L}{kg}\right) = \frac{\text{Ion concentration in plant tissue (fresh weight, FW) at harvest (mg/kg)}}{\text{Initial ion concentration in growth solution (mg/L)}} \quad (1)$$

2.5 Perchlorate Purification

The ClO₄⁻ in the Purolite® A530E resin columns was extracted and purified at Oak Ridge National Laboratory using procedures described previously (Hatzinger et al., 2011; Böhlke et al., 2017). The extraction process consisted of removing the adsorbed ClO₄⁻ from the resin in the Purolite® A530E column, rinsing it with ultra-pure DDI water, transferring it into a preparative glass chromatography column (100 mL volume), and then washing it with

approximately 5 bed volumes of 4 M HCl to remove impurities (i.e. NO_3^- , organics, carbonates) (Hatzinger et al., 2011; Böhlke et al., 2017). The ClO_4^- was eluted from the preparative glass column with a mixed solution of 1 M FeCl_3 and 4 M HCl. The FeCl_3 -HCl eluent solution (containing the eluted ClO_4^-) was neutralized with NaOH (pH $\sim 9 - 10$) to precipitate out Fe as $\text{Fe}(\text{OH})_3$ (Hatzinger et al., 2011; Böhlke et al., 2017). After removing $\text{Fe}(\text{OH})_3$ precipitates by centrifugation, the supernatant solution was collected and evaporated using a vacuum concentration system until the ClO_4^- concentration in solution was in excess of 3 mg/mL (Hatzinger et al., 2011; Böhlke et al., 2017). After removing salts (or NaCl precipitates during evaporation), the ClO_4^- in the remaining solution was crystallized as cesium perchlorate (CsClO_4) by adding CsCl (Hatzinger et al., 2011; Böhlke et al., 2017).

2.6 Isotopic Ratio Analyses

Stable isotope ratios of O and N in ClO_4^- and NO_3^- ($\delta^{18}\text{O}$, $\delta^{17}\text{O}$, $\Delta^{17}\text{O}$, and $\delta^{15}\text{N}$) in hydroponic solutions and plant extracts were analyzed at the Reston Stable Isotope Laboratory of the U.S. Geological Survey in Reston, VA (Sigman et al., 2001; Casciotti et al., 2002; Hatzinger et al., 2011; Böhlke et al., 2017); Selected samples were analyzed for NO_2^- concentration and isotopic composition, as NO_2^- can interfere with NO_3^- isotopic analysis (Böhlke et al., 2007; Casciotti et al., 2007). Oxygen stable isotope ratio analyses of ClO_4^- were performed by high-temperature conversion of the purified ClO_4^- salt (CsClO_4) to CO that was analyzed in continuous-flow mode by isotope-ratio mass spectrometry (CO-CFIRMS) and by in vacuo decomposition of the ClO_4^- salt (CsClO_4) to O_2 that was analyzed in dual-inlet mode by isotope-ratio mass spectrometry (O2-DIIRMS) (Hatzinger et al., 2011; Böhlke et al., 2017). Chlorine isotope ratios ($\delta^{37}\text{Cl}$) of ClO_4^- were analyzed at the Environmental Isotope Geochemistry Laboratory of the University of Illinois at Chicago by conversion of the CsClO_4 salt to methyl

chloride (CH₃Cl) gas that was analyzed by isotope-ratio mass spectrometry (IRMS) (Hatzinger et al., 2011; Böhlke et al., 2017).

Relative isotope ratios of samples are reported in delta notation (δ), defined as deviations from the corresponding molar ratios (R) in specified standards (std): $\delta^iE = R_{\text{sample}}/R_{\text{std}} - 1$, in parts per thousand (‰). For $\delta^{18}\text{O}$, R is $^{18}\text{O}/^{16}\text{O}$ and std is VSMOW; for $\delta^{17}\text{O}$, R is $^{17}\text{O}/^{16}\text{O}$ and std is VSMOW; for $\delta^{15}\text{N}$, R is $^{15}\text{N}/^{14}\text{N}$ and std is atmospheric N₂; for $\delta^{37}\text{Cl}$, R is $^{37}\text{Cl}/^{35}\text{Cl}$ and std is SMOC. Non-mass-dependent relative excess abundances of ^{17}O are given as $\Delta^{17}\text{O}$ values: $\Delta^{17}\text{O} = (\delta^{17}\text{O} + 1)/(\delta^{18}\text{O} + 1)^{0.525} - 1$, also in parts per thousand (‰). Secondary isotopic reference materials and calibration values used for ClO_4^- and NO_3^- have been reported previously (Böhlke et al., 2009; Böhlke et al., 2017; Böhlke et al., 2003) and are given in the SI.

Estimates of the N and O isotopic fractionation effects ($\epsilon^{15}\text{N}$ and $\epsilon^{18}\text{O}$, respectively) imparted on NO_3^- were derived by fitting data to linear Rayleigh equations 2–3, where $f \approx [\text{NO}_3^-] / [\text{NO}_3^-]_{\text{initial}}$ (Mariotti et al., 1981).

$$\ln(\delta^{15}\text{N} + 1) = \ln(\delta^{15}\text{N}_{\text{initial}} + 1) + \epsilon^{15}\text{N} \times \ln(f) \quad (2)$$

$$\ln(\delta^{18}\text{O} + 1) = \ln(\delta^{18}\text{O}_{\text{initial}} + 1) + \epsilon^{18}\text{O} \times \ln(f) \quad (3)$$

Regression of $\ln(\delta^{15}\text{N} + 1)$ or $\ln(\delta^{18}\text{O} + 1)$ on $\ln(f)$ yields slopes of $\epsilon^{15}\text{N}$ and $\epsilon^{18}\text{O}$, respectively (Mariotti et al., 1981). Regressions and associated statistical errors for isotope data and anion mass data were determined by using the regression wizard function of the Sigmaplot 11 Scientific Data Analysis and Graphing Software. Values of δ and ϵ defined above are reported in parts per thousand (per mil, ‰).

3 Results

3.1 Hydroponic Snap Bean Study

3.1.1 Changes in Hydroponic Growth Solutions

Substantial water loss occurred in both hydroponic experiments and was mainly attributed to transpiration. The cumulative volume of growth solution lost in treatments with no plants was less than 10% of the initial volume compared to cumulative losses of ~70 to 230 % of the initial volume in treatments with plants (SI Figure S1). Within each experiment, the volume of solution transpired by plant treatments was similar regardless of the presence or absence of ClO_4^- , but the volume transpired in experiment 2 was approximately twice that of experiment 1 (SI Figure S1). Experiment 1 was conducted during the months of October and December and experiment 2 during the months of April and May when temperatures were higher, which may have contributed to higher transpiration in experiment 2. Individual tubs contained more plants in experiment 1 (Table 1), but plant mass was 2-4 fold higher in experiment 2, also contributing to greater transpiration in experiment 2.

As in a previous study (Seyfferth and Parker, 2007) ClO_4^- mass in hydroponic solutions of treatments with plants decreased gradually over time in a non-linear fashion with a cumulative mass loss of 60-80% of the initial ClO_4^- solution mass (SI Figure S2). The fractional mass loss of ClO_4^- from solution was independent of initial ClO_4^- concentration, but there was a larger overall decrease in ClO_4^- solution mass in experiment 2, which had higher transpiration water loss (SI Figure S2). Linear correlations that were independent of initial ClO_4^- concentration ($r^2 = 0.82$ and 0.96 for 2 mg/L and 10 mg/L ClO_4^- , respectively, $P < 0.05$) were observed between the volume of solution transpired and the fraction of total ClO_4^- mass lost from solution in experiment 2 (Figure 1a). Insufficient time points were available to evaluate such correlations in experiment 1. Our results support previous work (Seyfferth and Parker, 2007) that suggests ClO_4^- mass lost from solution is directly proportional to the volume of transpired water, but less than the amount based on transpiration and solution ClO_4^- concentration (SI Figure S3).

Like ClO_4^- mass, the NO_3^- mass in solutions of treatments with plants gradually decreased ($\sim 40 - 80 \%$) over time in a non-linear fashion for all treatments with plants in both experiments (SI Figure S2). The NO_3^- fractional mass loss was linearly correlated ($P < 0.05$) with the amount of solution transpired in all treatments with plants ($r^2 = 0.76-0.86$) (Figure 1c). Both NO_3^- and ClO_4^- had similar uptake rates as % of total mass/L transpired (0.87 ± 0.15 to 0.99 ± 0.21) and (0.97 ± 0.08 to 1.02 ± 0.17), respectively, supporting a similar uptake mechanism as suggested by other studies (Figure 1a and c) (Seyfferth et al., 2008a).

In contrast to ClO_4^- and NO_3^- , Cl^- mass in solution remained relatively constant (~ 80 - 120% of initial total Cl^-) throughout the experiments regardless of initial ClO_4^- treatment concentration, the presence or absence of plants, or Cl^- concentration (e.g. experiment 1 $\text{Cl}^- \approx 1$ mg/L and experiment 2 $\text{Cl}^- \approx 100$ mg/L) (SI Figure S2). There was no correlation between mass of Cl^- removed from solution and the amount of solution transpired (Figure 1b). Although Cl^- clearly accumulated in plants (Table S1), the amount taken up was small compared to the amount in solution (experiment 2) or small compared to analytical uncertainty (experiment 2).

3.1.2 Anion Distributions and Bioconcentration factors

Bioconcentration factors (BCF, Equation 1) for ClO_4^- ranged from $55 - 70$ L/kg for all ClO_4^- exposure concentrations in both experiments, with the low and high values in this range corresponding to the 10 mg/L ClO_4^- treatments of experiment 2 and experiment 1, respectively. Our ClO_4^- BCF values were higher than those reported for lettuce exposed to 1.25 and 10 $\mu\text{g/L}$ ClO_4^- ($2 - 40$ L/kg) (Seyfferth and Parker, 2007) but within range of those reported for spinach ($17 - 102$ L/kg) (Ha et al., 2011) exposed to $4 - 40$ $\mu\text{g/L}$ ClO_4^- . The distribution of ClO_4^- between different plant tissues was independent of ClO_4^- exposure concentration in both experiments (SI Table S1). Of the ClO_4^- found in the plant, the majority was located in leaves ($77 - 90\%$), a finding consistent with past studies (Jackson et al., 2005; Seyfferth and Parker,

2007; Voogt and Jackson, 2010). The next largest accumulation of ClO_4^- was detected in bean pods (5 – 21%), followed by stems (1.2 – 2.8%), and then roots (1 – 2%).

Apparent BCF factors for NO_3^- for all exposure concentrations in both experiments (0.5–2 L/kg) were much smaller than the ClO_4^- BCF values because most of the NO_3^- was converted within plants to organic N. Only a small fraction of the initial total NO_3^- mass was detected in plant tissues for both experiments (0.3 – 5.0 %), with the accumulation being higher in experiment 2 (SI Table S1). Accumulated NO_3^- was mainly located in the roots (18 – 45 %), followed by the leaves (22 – 39%), the stems (14 – 33 %), and then bean pods (3 – 27 %). In experiment 1, in treatments with plants, most of the ~ 41 – 45 % NO_3^- (as N) that was lost from solution was recovered as N in plant tissues, as indicated by nearly complete recovery of the initial total N (SI Table S1). Experiment 1 BCF average values for total N were 158–181 L/kg for all plant treatments. Total N was not measured in experiment 2. The majority of total N accumulated in the plant was located in the bean pods (39 – 44 %) and the leaves (34 – 43 %), followed by the stems (10 – 12 %), and then roots (8 – 10 %).

The BCF values for Cl^- (1.9–8.8 L/kg FW) in experiment 2 were much lower than those for ClO_4^- , supporting the selective uptake of ClO_4^- . The small amount of Cl^- extracted from the plants was mainly found in the stems (34 – 43 %), followed by the leaves (24 – 42 %), the bean pods (10 – 23 %), and then roots (9 – 24 %) (SI Table S1).

3.1.3 Isotopic Composition of Perchlorate

The isotopic composition of ClO_4^- in both the growth solutions and plant extracts were measured at plant maturity and compared to the initial isotopic composition of ClO_4^- in the growth solutions to evaluate any fractionation associated with either (1) uptake from solutions into the plants or (2) transformation or exchange within the plants after uptake. The isotopic compositions of ClO_4^- ($\delta^{37}\text{Cl}$, $\delta^{18}\text{O}$, $\Delta^{17}\text{O}$) in leaves and hydroponic solutions were not

consistently different from each other in either experiment using isotopically different ClO_4^- sources ($n = 4$ pairs) (Figure 2). For most experiments using isotopically anomalous ClO_4^- (Figure 2a and 2b), small differences (< 1.5 ‰) between final growth solutions and leaf extracts were not systematically in one direction or the other, and likely represent small random variations in impurities or isotope effects of sample preparation (Sturchio et al., 2006; Böhlke et al., 2017). Experiments using isotopically anomalous ClO_4^- also exhibited small systematic differences between the $\delta^{18}\text{O}$ and $\Delta^{17}\text{O}$ values of the initial ClO_4^- reagent and final hydroponic solutions (1-2 ‰). Those offsets are not consistent with fractionation effects and are attributed to minor contaminants in the solution extract samples (Böhlke et al., 2017), which have much less effect on the results of experiments using isotopically normal ClO_4^- reagent (Figure 2c and 2d). One of the 2 mg/L ClO_4^- replicate treatments in experiment 1 had anomalously high $\delta^{18}\text{O}$ (~ 7 ‰ above the initial value) and $\delta^{37}\text{Cl}$ ($\sim 1 - 2$ ‰ above the initial value) in leaf tissue, but no difference in $\Delta^{17}\text{O}$ (Figure 2a and 2b). That sample may have been isotopically fractionated (e.g., partially degraded), but it is not known if the anomalous result was caused by plant processes or subsequent handling of the organic-rich extract. The NO_3^- extracted from that sample was not isotopically anomalous compared to others in the same experiment. For treatments with normal reagent ClO_4^- (Figure 2c and 2d), small variations in $\delta^{37}\text{Cl}$ and $\delta^{18}\text{O}$ could be consistent with fractionation effects in growth solutions or plants, but overall variations were ≤ 1 ‰, which is similar to typical uncertainties of approximately ± 0.5 ‰ to ± 1.0 ‰ due to combined effects of ClO_4^- extraction, purification, and isotopic analysis of complex solutions. Overall, the data from both experiments indicate generally insignificant isotopic fractionation or exchange attributable to plant ClO_4^- uptake, plant ClO_4^- transformation, microbial ClO_4^- transformation, or isotopic exchange between ClO_4^- and other constituents.

3.1.4 Isotopic Composition of Nitrate

The $\delta^{15}\text{N}$ values of NO_3^- in hydroponic solutions increased slightly ($< 2 \text{ ‰}$) over time in both experiments for all treatments with plants but no increase was observed in control treatments without plants (Figure 3a and c). In experiment 1, $\delta^{15}\text{N}$ increases were small but fairly constant throughout the growth period (Figure 3a), whereas in experiment 2 the increases did not occur until mid-way through the growth period (Figure 3c). In the two treatments with the largest $\delta^{15}\text{N}$ increases, $\delta^{18}\text{O}$ also increased substantially (Figure 3d), whereas $\delta^{18}\text{O}$ variations in other treatments were within normal analytical uncertainties (Figure 3b). Apparent isotope fractionation factors (ϵ) derived from residual NO_3^- in both experiment growth solutions were of the order of -1.0 to -1.5 ‰ for $\epsilon^{15}\text{N}$ ($P < 0.05$) (SI Figure S4). The $\epsilon^{18}\text{O}$ values were generally insignificant ($P > 0.05$) in both experiment solutions. Correlated increases in $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in late stages (last ten days) of two treatments in experiment 2 could indicate minor fractionation by plant uptake or NO_3^- reduction. Apparent $\epsilon^{15}\text{N}/\epsilon^{18}\text{O}$ ratio values for NO_3^- in solutions of approximately $0.5 - 0.6$ were lower than reported experimental values for NO_3^- reduction ($\epsilon^{15}\text{N}/\epsilon^{18}\text{O} \approx 1$) and more like those reported for NO_3^- transport from solution into plants (Needoba et al., 2003; Needoba et al., 2004; Granger et al., 2010) (SI Figures S4 and S5).

The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of NO_3^- extracted from leaves were higher than those in the hydroponic solutions by about $10\text{-}20\text{ ‰}$ (Figure 4). These differences were similar in both experiments, even though the NO_3^- reagents used in the experiments had different initial isotopic compositions (Figure 4), consistent with isotopic fractionation of NO_3^- within the plants after uptake from growth solutions (Schmidt et al., 2015; Evans, 2001; Liu et al., 2012). In both experiments there were positive ($P < 0.001$) correlations between $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of leaves and growth solutions with slopes of 1.06 (experiment 1) and 1.04 (experiment 2). When evaluated using Equations 7 and 8, data from both experiments yielded $\epsilon^{15}\text{N}/\epsilon^{18}\text{O}$ ratios of 1.05 ,

consistent with reported effects of NO_3^- reduction during assimilation by plants (Granger et al., 2004; Casciotti et al., 2002). In experiment 1, $\delta^{15}\text{N}$ values were $\sim +54\text{‰}$ for solution NO_3^- , $\sim +31\text{‰}$ for final plant tissue, and $\sim +72\text{‰}$ for final residual NO_3^- extracted from leaves (Figure 4a). These results are qualitatively consistent with isotope effects dominated by internal NO_3^- reduction during assimilation (Karsh et al., 2012; Granger et al., 2004; Karsh et al., 2014).

3.2 Snap Bean Field Studies

In 2010, the isotopic composition of ClO_4^- in the Raleigh, NC snap bean crops was indistinguishable, given cumulative error terms ($< \pm 2\text{‰}$), from that of ClO_4^- in the groundwater used for irrigation (SI Table S3). The isotopic composition of ClO_4^- in the groundwater was similar to that of ClO_4^- from the Atacama Desert, indicating that a likely source of elevated ClO_4^- in the groundwater was from the historical use of imported Chilean NO_3^- fertilizer (Böhlke et al., 2009; Böhlke et al., 2005). These data indicate the 2010 Raleigh snap bean crop accumulated Atacama-derived ClO_4^- from irrigation water without fractionating or otherwise altering its isotopic composition substantially, thus supporting the hydroponic results in a field setting. For ClO_4^- from the 2009 snap bean crop, all three isotopic parameters ($\delta^{37}\text{Cl}$, $\delta^{18}\text{O}$, and $\Delta^{17}\text{O}$) were shifted slightly away from the 2010 values and plot near two-component mixing lines between the irrigation water data and values commonly attributed to indigenous natural ClO_4^- from uncontaminated soils and groundwaters in other areas of the U.S. (Figure 5)(Jackson et al., 2010; Poghosyan et al., 2014).

Differences in isotopic composition of ClO_4^- accumulated in snap beans in Raleigh, NC in 2009 and 2010 are qualitatively consistent with estimated differences in the masses of applied ClO_4^- from irrigation water and atmospheric deposition between years (SI Table S3). The snap bean crop received varying proportions of rainfall and groundwater irrigation in 2009 and 2010.

Concentrations of ClO_4^- in irrigation water also varied between 2009 and 2010 (~ 0.6 and 2.2 $\mu\text{g/L}$, respectively), presumably due to exhaustion of the ion-exchange column installed to remove ClO_4^- from the groundwater used for irrigation. Although partial retention of ClO_4^- on the resin could have caused some isotopic fractionation of ClO_4^- in the irrigation water, previous studies indicate those effects are small compared to the differences observed in the Raleigh, NC study (Hatzinger et al., 2011; Böhlke et al., 2017). Furthermore, differences between 2009 and 2010 isotopic data included a substantial change in $\Delta^{17}\text{O}$, which would be consistent with different sources but not with variable fractionation. The similarity between the snap bean and groundwater isotopic composition in 2010 is consistent with the much larger applied ClO_4^- mass from groundwater during the growing season in 2010, (480 $\mu\text{g/m}^2\text{-yr}$) compared to 2009 (48 $\mu\text{g/m}^2\text{-yr}$) (SI Table S2). The intermediate isotopic composition of foliar ClO_4^- in 2009 is consistent with a larger proportional contribution from indigenous ClO_4^- in atmospheric deposition when the groundwater ClO_4^- contribution was smaller. The annual average ClO_4^- deposition rate in the US is ~ 6.5 $\mu\text{g/m}^2\text{-yr}$ in precipitation, with an unknown contribution from dry deposition (Rajagopalan et al., 2009). The maximum ClO_4^- contribution from fertilizer was estimated to be less than 2.5 $\mu\text{g/m}^2$. The higher mass of total ClO_4^- applied from both sources in 2010 is also reflected in a ~ 4X higher foliar ClO_4^- concentration in 2010 (3000 – 4000 $\mu\text{g/kg}$ dry weight) than 2009 (800 – 1000 $\mu\text{g/kg}$ dry weight).

The isotopic composition of ClO_4^- in snap beans from Long Island appears to reflect multiple sources, possibly dominated by indigenous ClO_4^- and Atacama ClO_4^- with a smaller contribution of synthetic ClO_4^- (Figure 5). It is not possible to determine if the foliar ClO_4^- isotopic composition is consistent with the contributing sources as no data are available for the isotopic composition of groundwater used for irrigation. Long Island groundwater has

previously been shown to contain varying proportions of synthetic ClO_4^- and Atacama ClO_4^- in some areas where ClO_4^- concentrations are relatively high (Böhlke et al., 2009). Given the low concentration of ClO_4^- in groundwater used for irrigation at the Long Island experiment location ($\sim 0.3 \mu\text{g/L}$), any combination of two or more sources would not be unreasonable. Similar ClO_4^- isotopic compositions have been reported for groundwater with similarly low ClO_4^- concentrations from mixed sources in southern California (Hatzinger et al., 2015; Sturchio et al., 2014). Our data indicate that it may be possible to evaluate foliar ClO_4^- isotopic composition and origin in field-grown plants, even with near-background foliar ClO_4^- concentrations ($< 400 \mu\text{g/kg}$ dry weight).

3.3 Spinach Study Results

We analyzed commercially grown spinach as another test of our extraction and analysis procedures and to evaluate potential sources of ClO_4^- in food products. The isotopic composition of ClO_4^- extracted from spinach ($\delta^{37}\text{Cl} = + 3.3 \text{ ‰}$, $\delta^{18}\text{O} = + 3.7 \text{ ‰}$, $\Delta^{17}\text{O} = + 2.6 \text{ ‰}$) was similar to that reported for indigenous natural ClO_4^- in soil and groundwater in the southwestern U.S. and surface waters of the Great Lakes, and to the inferred isotopic composition of natural ClO_4^- in groundwater in parts of southern California (Figure 5) (Jackson et al., 2010; Böhlke et al., 2005; Hatzinger et al., 2015; Sturchio et al., 2006; Sturchio et al., 2014; Poghosyan et al., 2014).

4 Discussion

A number of processes could affect the stable isotopic composition of ClO_4^- accumulated in plants and potentially contribute to the reported ranges of natural ClO_4^- stable isotopic composition in soil and groundwater. Uptake of ClO_4^- by ion transporters, plant-catalyzed reduction, or oxygen exchange could all potentially cause isotopic fractionation. Our study is the first to directly use stable isotopic analyses of ClO_4^- extracted from plant tissues to evaluate the potential of plant processes to alter ClO_4^- through fractionation or exchange.

Snap bean plants were found to selectively uptake ClO_4^- relative to Cl^- , but not relative to NO_3^- , supporting a common ion transport mechanism for ClO_4^- and NO_3^- . The similarity in uptake rates for ClO_4^- and NO_3^- , the strong correlation of anion uptake with volume of transpired solution, and the partial exclusion of ClO_4^- relative to the mass of transpired water, are all consistent with findings from a previous study (Seyfferth et al., 2008a). This suggests that transport is through an ion carrier in the roots that is specific to both NO_3^- and ClO_4^- but is also dependent on transpiration rate (Seyfferth et al., 2008a).

No substantial differences were observed in the $\delta^{37}\text{Cl}$, $\delta^{18}\text{O}$, or $\Delta^{17}\text{O}$ values of ClO_4^- between the growth solutions and leaf extracts during hydroponic experiments despite a large isotopic fractionation of residual NO_3^- in leaf extracts. Changes in $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of NO_3^- in growth solutions were small but measurable in two treatments. If minor isotopic fractionation by bacterial NO_3^- reduction or transport into plants was responsible for these changes in growth solutions, those processes did not have a measurable effect on residual ClO_4^- in the growth solutions. Although some of the ClO_4^- mass in experiment 1 was not accounted for, data from experiment 2 indicate ClO_4^- mass was largely conserved. Furthermore, the essentially identical stable isotopic compositions of ClO_4^- in source reagents, growth solutions, and leaf tissues in both experiments makes it unlikely that substantial transformation of ClO_4^- occurred, as previous studies indicate enzymatic ClO_4^- reduction is accompanied by large isotope fractionation effects (Sturchio et al., 2007; Hatzinger et al., 2009; Sturchio et al., 2003). Our results, in combination with another published study (Seyfferth 2008b), suggest that plant-mediated ClO_4^- transformation may not be common. If isotopic fractionation of ClO_4^- occurred during uptake or if plant-mediated exchange of O occurred between ClO_4^- and other compounds such as H_2O ,

then the magnitude of exchange was too small to detect in these experiments (i.e., $\leq 2\%$ exchange in a growing season).

In contrast to ClO_4^- , $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of NO_3^- in plant tissue was fractionated substantially (by about 10 – 20 ‰, relative to NO_3^- in growth solutions), consistent with reported effects of NO_3^- assimilation (Needoba et al., 2004; Granger et al., 2010; Ledgard et al., 1985). The $\epsilon^{15}\text{N}/\epsilon^{18}\text{O}$ ratios of 1.05 observed in our study support previous experimental studies indicating that isotopic fractionation by assimilatory nitrate reductase has an intrinsic $\epsilon^{15}\text{N}/\epsilon^{18}\text{O}$ ratio of approximately 1, regardless of the magnitudes of $\epsilon^{15}\text{N}$ and $\epsilon^{18}\text{O}$ values expressed (Karsh et al., 2012; Granger et al., 2004; Karsh et al., 2014).

The isotope effects of assimilation and storage of NO_3^- in plant tissue, as well as isotope effects attributed to both uptake and efflux of NO_3^- , are not all known, but our study provides information that could improve applicability of plant leachate NO_3^- isotopes as indicators of fertilization history. Recent studies (Laursen et al., 2013; Mihailova et al., 2014) have suggested that isotopic analyses of NO_3^- extracted from plants could be used to distinguish between organically and conventionally grown crops (Laursen et al., 2013; Mihailova et al., 2014). The O and N fractionation ratio ($\epsilon^{15}\text{N}/\epsilon^{18}\text{O} \approx 1$) in our study could be used to constrain the isotopic composition of a fertilizer source after it has undergone varying amounts of fractionation in plants. Furthermore, because ClO_4^- has been a component of some natural NO_3^- fertilizers, various combinations of NO_3^- isotopes, ClO_4^- concentrations, and ClO_4^- isotopes in plant extracts may be useful in identifying natural NO_3^- fertilizer components. For instance, natural NO_3^- fertilizer with high $\delta^{18}\text{O}$ and $\Delta^{17}\text{O}$ from the Atacama Desert might be recognized from its unusual isotopic composition (Böhlke et al., 1997; Michalski et al., 2004). Even where Atacama NO_3^- only makes up a small fraction of total N applications, it can contain substantial amounts of

ClO_4^- . Therefore, ClO_4^- concentrations and isotope ratios could also be used as a marker to detect the use of natural inorganic NO_3^- fertilizers from the Atacama Desert (Böhlke et al., 2009) or the presence of ClO_4^- in irrigation water (this study).

Our study indicates that some field-grown plants exposed to environmentally relevant ClO_4^- concentrations can accumulate ClO_4^- in leaf tissues without substantial isotopic alteration. Snap bean crops grown in Raleigh, NC and Long Island, NY had ClO_4^- isotopic compositions consistent with known or inferred local sources including irrigation water and atmospheric deposition. ClO_4^- extracted from commercial spinach grown in the western United States had an isotopic composition similar to that of indigenous natural ClO_4^- from the southwestern United States and the Great Lakes (Jackson et al., 2010; Sturchio et al., 2014; Poghosyan et al., 2014). The low concentrations of ClO_4^- in the spinach are consistent with exposure to low concentrations of indigenous natural ClO_4^- previously reported in soil and groundwater (Jackson et al., 2010; Jackson et al., 2015). Although this spinach represents only one composite sample, and we have no direct knowledge about ClO_4^- in fertilizer or irrigation water used to grow the spinach, these results, combined with those of the field experiments and hydroponic studies with snap beans, suggest that it may be possible to evaluate the source of ClO_4^- in some types of plants including commercial produce and in other plant-based food products. This finding is important because most of the exposure to ClO_4^- in the U.S. population, and likely the population of many other countries, is through produce (Jackson et al., 2005; Sanchez et al., 2005). Isotopic analyses of ClO_4^- in plants is a useful method for characterizing the isotopic composition of ClO_4^- in the irrigation water or fertilizer used for cultivation. Locations and times at which produce is grown are not always known. Fertilizers, irrigation water sources, and/or sources of ClO_4^- in irrigation water or soils can vary in a given location and some produce is from multiple

growers or is imported from other countries. Thus, isotope-ratio measurements of plant tissue provide a direct method to distinguish the dominant sources of ClO_4^- in the food chain. Our results also have implications in the field of phytoremediation as ClO_4^- isotopes might be useful for assessing whether plants are taking up targeted contaminant sources and would help improve the management and disposal of phytoremediation products if the ClO_4^- is not broken down and can be returned to the soil and groundwater when plants turnover. In addition, our study illustrates that testing of irrigation water supplies may be important for minimizing ClO_4^- contamination of produce. In areas where irrigation wells tap groundwater containing legacy ClO_4^- from agricultural use of natural nitrate fertilizers from the Atacama Desert (as in our Raleigh, NC study), it may be possible either to treat it, or to switch to shallower (younger) or deeper (older) groundwater sources that were recharged when ClO_4^- loading from those fertilizers was less (Böhlke et al., 2009).

It is possible that hydroponic studies may not fully reflect uptake processes in normal soil-plant systems and that snap beans may not represent other plant types. However, our results indicate ClO_4^- uptake and accumulation can occur without significant net loss or isotope effects and that plant NO_3^- reduction systems efficiently exclude ClO_4^- , in contrast to some microbial NO_3^- reducing systems that can reduce both NO_3^- and ClO_4^- (Hatzinger et al., 2009). These results suggest that isotopic characteristics of ClO_4^- in soils and groundwater may not be affected by plant uptake and release. Therefore, our results contradict the hypothesis that plant uptake is a major cause of variation in ClO_4^- stable isotopic composition observed in natural systems. Given the relatively large contribution of U.S. human exposure through food, further studies may be warranted to determine sources of ClO_4^- in the food chain, as well as to determine if other types of plants in other settings can alter the isotopic composition of ClO_4^- .

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6 Supporting Information Available

An Excel workbook containing primary data for hydroponic experiments, plus additional Tables S1-S3, and additional Figures S1-S5 are provided.

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Table 1. Summary of initial experimental conditions for ClO_4^- treatments in experiment 1 and 2. Experiment 1 was conducted during the months of October and December while experiment 2 was conducted during April and May.

Parameter	Experiment 1				Experiment 2			
	ClO_4^- Treatments (mg/L)				ClO_4^- Treatments (mg/L)			
	0	1	2	10	0	1	0.01	10
Cl ⁻ (mg/L)	1.2 (0)	1.0 (0)	1.3 (0.4)	1.7 (0.3)	103 (0)	105 (0)	108 (1)	108 (3)
NO ₃ ⁻ -N (mg/L)	274 (0)	268 (0)	274 (3)	264 (13)	237 (0)	240 (0)	237 (2)	235 (4)
PO ₄ ³⁻ (mg/L)	48 (0)	46 (0)	72 (17)	59 (23)	72 (0)	74 (0)	76 (5)	70 (1)
SO ₄ ²⁻ (mg/L)	117 (0)	110 (0)	116 (2)	113 (9)	130 (0)	137 (0)	144 (12)	142 (15)
# tubs (replicates)	1	1	4	2	1	1	2	2
Plants per tub	6	0	6	6	4	0	4	4

Anion concentrations reported are the means of the replicates for each ClO_4^- treatment. Standard errors of the means are indicated in parenthesis (e.g. Mean ($\pm$ SD)).

Figure 1. Relation between fraction of initial total (a) ClO_4^- , (b) Cl^- , and (c) NO_3^- mass lost from solution and cumulative volume of solution transpired over the course of experiment 2. Symbols with error bars indicate average values ($\pm$ standard deviation) for replicated treatments ($n = 2$ for 0.01 and 10 mg/L ClO_4^- and $n = 1$ for 1 mg/L and no ClO_4^- controls). Regressions and associated errors were calculated using the regression wizard function of the Sigmaplot 11 graphing software. The colors of regression lines and regression data are the same as those of the symbols for the treatments they represent (e.g. dark blue = 10 mg/L ClO_4^-). Not enough data were collected for a similar analysis of experiment 1. All ClO_4^- treatments had plants except the 1 mg/L ClO_4^- control.

Figure 2. Stable isotopic compositions of ClO_4^- in nutrient solutions and leaf extracts of all replicate treatments in experiment 1 and 2. Leaf extracts are represented by the open symbols while the final growth solutions are represented by the solid symbols. Experiment 1 replicate treatments are represented by the red triangles ($n = 4$), green circles ($n = 2$), and light blue cross symbols ($n = 1$), while experiment 2 replicates are represented by the dark blue squares ($n = 2$) and pink diamond symbols ($n = 1$), where ‘ n ’ equals the number of replicates. $\delta^{37}\text{Cl}$, $\delta^{18}\text{O}$, and $\Delta^{17}\text{O}$ values are referenced to 0 for Standard Mean Ocean Chloride (SMOC) and Vienna-Standard Mean Ocean Water (VSMOW) international reference standards, respectively.

Figure 3. Stable isotopic compositions of NO_3^- in nutrient growth solutions as a function of time. Symbols with error bars indicate average values ($\pm$ standard deviation) for replicated treatments ($n = 2$ for 0.01 and 10 mg/L ClO_4^- and $n = 1$ for 1 mg/L and no ClO_4^- controls). In experiment 1 the NO_3^- isotopic compositions of growth solutions in control treatments were not measured and the NO_3^- isotopic compositions of growth solutions of only one replicate in the 2 and 10 mg/L ClO_4^- treatments were measured. First time point in each experiment represents the NO_3^- isotopic composition of initial solution. All ClO_4^- treatments had plants except the 1 mg/L ClO_4^- no plants control.

Figure 4. Stable isotopic compositions of NO_3^- in final growth solutions (solid symbols) and leaf extracts (open symbols) of treatments with plants. Symbols with error bars indicate average values ($\pm$ standard deviation) for replicated treatments: $n = 4$ for 2 mg/L ClO_4^- (experiment 1), $n = 2$ for 0.01 and 10 mg/L ClO_4^- (experiment 1 and 2), and $n = 1$ for 1 mg/L and no ClO_4^- controls (experiment 1 and 2). Plant total-N $\delta^{15}\text{N}$ was only measured in experiment 1. The regression slope is equal to the $\Delta(\delta^{15}\text{N}:\delta^{18}\text{O})$ ratio. Note that the standard deviations shown are smaller than the symbol size.

Figure 5. Summary of isotope data for ClO_4^- from Raleigh, NC and Long Island, NY field studies and commercial spinach displayed with previously published ClO_4^- isotope data from synthetic (electrochemical) and natural sources from the Atacama Desert in Chile, Death Valley (DV), California, and the Southern High Plains (SHP) and Middle Rio Grande Basin (MRGB) of Texas and New Mexico (Jackson et al., 2010; Böhlke et al., 2005). Symbols with error bars for the 2010 Raleigh bean varieties S156 ($n = 3$) and R123 ($n = 2$) indicate average values ($\pm$ standard deviation). The mixing line indicates varying proportions of two end-member ClO_4^- sources (Raleigh groundwater and natural RGB/SHP-type), with hash lines at 10% intervals. Symbols for S156-2010 Raleigh Beans are largely masked by those of R123-2010 Raleigh Beans.