Using Respiration Quotients to Track Changing Sources of Soil Respiration Seasonally and with Experimental Warming

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Abstract. Developing a more mechanistic understanding of soil respiration is hampered by the difficulty in determining the contribution of different organic substrates to respiration and in disentangling autotrophic versus heterotrophic and aerobic versus anaerobic processes. Here, we use a relatively novel tool for better understanding soil respiration: the apparent respiration quotient (ARQ). ARQ is the amount of CO_2 produced in the soil divided by the amount of O_2 consumed and it changes according to which organic substrates are being consumed and whether oxygen is being used as an electron acceptor. We investigated how the ARQ of soil gas varied seasonally, by soil depth, and by *in situ* experimental warming (+4°C) in a coniferous forest whole-soil-profile warming experiment over two years. We then compared the patterns in ARQ to those of soil $\delta^{13}CO_2$. Our measurements showed strong seasonal variations in ARQ from ≈0.9 during the late spring and summer to ≈0.7 during the winter. This pattern likely reflected a shift from respiration being fueled by oxidized substrates like sugars and organic acids derived from root and root respiration during the growing season to more reduced substrates such as lipids and proteins derived from microbial necromass during the winter. This interpretation was supported by $\delta^{13}CO_2$ values, which were relatively lower, like lipids, in the winter and relatively higher, like sugars, in the summer. Furthermore, experimental warming significantly changed how both ARQ and $\delta^{13}CO_2$ responded to soil temperature. Wintertime ARQ and $\delta^{13}CO_2$ values were higher in heated than in control plots, probably due to the warming-driven increase in microbial activity that may have utilized oxidized carbon substrates, while growing season values were lower in heated plots. Experimental warming and phenology change the sources of soil respiration throughout the soil profile. The sensitivity of ARQ to these changes demonstrates its potential as a tool for disentangling the biological sources contributing to soil respiration.

1 Introduction

Despite making extensive measurements of soil respiration (Bond-Lamberty and Thomson, 2010), scientists lack methods to disentangle the processes underlying, and substrates contributing to, soil respiration, which hampers predictions of terrestrial carbon cycle responses to global change (Phillips et al., 2017). Mechanistic uncertainty surrounding soil respiration is partly responsible for the 1000 Pg spread in model predictions of end-of-century terrestrial carbon-climate feedbacks (Friedlingstein et al., 2013). Soil respiration is the flux of CO_2 from the soil surface to the atmosphere, which is dominated by autotrophic respiration from plant roots and heterotrophic respiration from soil microbes. Heterotrophic respiration, which has increased globally over the past three decades (Bond-Lamberty et al., 2018), is itself the sum of various processes using different sources of energy. For example, microbes consume different organic substrates depending on what molecules are accessible and whether the microbes are living in the rhizosphere or bulk soil, and microbes utilize different terminal electron acceptors depending on O_2 availability in the microsites in which they reside (Keiluweit et al., 2016; Liptzin et al., 2011). The electron donors (the organic substrates) and the electron acceptors used by soil microbes during respiration cannot be resolved by measuring the CO_2 flux alone. Previous studies have used measurements of $\delta^{13}C$ to partition respiration into autotrophic and heterotrophic components (e.g., Dorrepaal et al., 2009), radiocarbon to partition respiration sources by age (e.g., Trumbore, 2000), or both isotopes in combination to more finely separate respiration among sources (e.g., Hicks Pries et al., 2013; Hopkins et al., 2012). However, isotopes are not the only way to disentangle soil respiration's various components (Subke et al., 2006).

Our ability to understand soil respiration is limited by measuring only one half of the respiration equation, the CO_2 produced. Simultaneously measuring the O_2 consumed can provide a more mechanistic understanding of the processes and substrates contributing to soil respiration (Phillips et al., 2017). The paired measurements of CO_2 and O_2 can be used to calculate a respiration quotient (RQ; Angert and Sherer, 2011). All organic matter has an oxidative ratio (1/RQ), which can be calculated based on an elemental analysis of its C, H, O, and N (Masiello et al., 2008). The oxidation state of carbon in carbohydrates is 0 with a corresponding RQ of 1 based on its elemental structure. More reduced energy sources such as lipids have lower RQ values (≈0.73) and the RQ of proteins range from 0.67 to 1; more oxidized sources such as organic acids have RQ ranges from 1 to 4 (Masiello et al., 2008; Table 1). The RQ of aerobic respiration therefore changes based on what substrates are being consumed (Dilly, 2001; Theenhaus et al., 1997). Anaerobic respiration increases RQ to values greater than one, as electron acceptors like Fe(III) and NO_3^- replace O_2. Thus, RQ can help differentiate between the electron donors (organic substrates) and terminal electron acceptors used during soil respiration. We will refer to the 'apparent' respiration quotient (ARQ) because not all ecosystem CO_2 or O_2 fluxes are due to respiratory processes (Angert and Sherer, 2011). For example, fluctuating redox conditions can lead to consumption of O_2 during metal oxidation and drive ARQ below the value of the most reduced organic matter (Angert et al., 2015).

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Thus far, CO_2:O_2 ratios have been primarily used to understand large scale earth system processes and only few studies have examined processes within ecosystems. This ratio in atmospheric samples has been used to estimate a) the magnitude of the terrestrial carbon sink, because carbon uptake by terrestrial ecosystems is balanced by O_2 production whereas ocean CO_2 uptake is decoupled from O_2, (Keeling, 1988; Keeling et al., 1996; Randerson et al., 2006; Worrall et al., 2013) and b) anthropogenic impacts on the carbon cycle, based on the principle that burning of reduced fossil fuels results in a different oxidative ratio than does photosynthesis and subsequent respiration of carbohydrates (Keeling, 1988). The CO_2:O_2 ratio of ecosystem-atmosphere exchanges is an essential quantity in these carbon cycle calculations. CO_2:O_2 ratios have been estimated from measurements of net ecosystem exchange of CO_2 and O_2 (e.g., Seibt et al., 2004) and from elemental analysis of biomass (Hockaday William C. et al., 2015; e.g., Masiello et al., 2008), both of which are assumed to be similar over multiyear timescales. In early carbon sink calculations, the oxidative ratio of ecosystem fluxes was assumed to be 1.1 (ARQ=0.9) based on a single study of temperate soils (Severinghaus, 1995). However, the few subsequent studies examining the CO_2:O_2 ratio of soil respiration fluxes have shown soil fluxes can deviate widely from that value.

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Soil ARQ from incubations shift as a result of temperature changes, substrate additions, and soil management. For example, the ARQ of peat soils decreased from about 1.1 to about 0.6 when temperatures increased from 0 to 20°C, attributed to changing substrate use (Chapman and Thurlow, 1998). Glucose additions to German forest soils increased soil ARQ to 0.95-1.0 from a basal value around 0.7 (Dilly, 2001; Theenhaus et al., 1997). Soils under organic agriculture were found to have a greater ARQ (1.19) than the same soils under conventional agriculture (0.72; Theenhaus et al., 1997). Soil ARQ in mesocosms containing pine seedlings changed seasonally and when the pine seedlings were cut, indicating the ratio is responsive to changes in vegetation (Andersen and Scagel, 1997; Scagel and Andersen, 1997).

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Lastly, in one of the only studies using *in situ* measurements, soil ARQ taken from gas wells across multiple forested ecosystems ranged widely from 0.14 to 1.23 indicating the influence of abiotic processes that consume O_2 (Angert et al. 2015). The wide range in soil ARQ values associated with different biochemical conditions indicates the ratio has the potential to provide insight into the substrates contributing to respiration as well as into abiotic O_2 consumption. Finer scale research is needed, however, to explore ARQ values in the same soils under different conditions to learn what these values indicate about the processes and substrates contributing to soil respired CO_2.

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Here we investigated how the ARQ of soil gas *in situ* varied seasonally, by soil depth, and by experimental warming in a whole-soil-profile warming experiment in a well-drained, oxygenated coniferous forest soil (Hicks Pries et al., 2017). We characterized soil ARQ at 30 and 90 cm depths in the winter and growing season over two years and compared the patterns in ARQ to monthly patterns in soil profile $\delta^{13}CO_2$. We hypothesized that ARQ values would change seasonally and with warming reflecting the values of the organic carbon substrates being consumed by microbes. Like ARQ, the $\delta^{13}C$ of soil CO_2 is

influenced by the use of different organic substrates since more reduced substrates tend to have lower $\delta^{13}C$ values (Bowling et al., 2008). By comparing ARQ values to other indicators of respiration sources, such as $\delta^{13}C$, augmented by what we understand about plant allocation of carbon substrates belowground, we aim to advance the utility of ARQ as a tracer of respiration processes.

2 Methods

2.1 Warming Experiment

The whole soil profile warming experiment is located at the University of California Blodgett Forest Research Station, in the Sierra Nevada foothills near Georgetown, CA at 1370 m above sea level. Mean annual precipitation is 1774 mm with most of it occurring from November through April and mean annual temperature is about 12.5°C (Bird and Torn, 2006). The experiment is in a thinned 80-year-old stand of mixed conifers including ponderosa pine (*Pinus ponderosa*), sugar pine (*Pinus lambertiana*), incense cedar (*Calodefrus decurrens*), white fir (*Abies concolor*), and douglas fir (*Pseudotsuga menziesii*). The soils are Holland series: fine-loamy, mixed, superactive, mesic Ultic Haploxeralfs of granitic origin with thick, >5 cm O horizons, minimal carbonates (Rasmussen et al., 2005), and a pH that ranges from 5.6 to 6.5 (Hicks Pries et al., 2018). The warming treatment warmed the soil +4°C to 1 m depth while maintaining the natural temperature gradient with depth and temporal variations in soil temperature as described in Hicks Pries et al. (2017). Briefly, there were three pairs of control and heated 3 m diameter circular plots heated by 22 vertical resistance heater cables in metal conduit (BriskHeat, Ohio, USA) that surrounded them. To compensate for surface heat loss, two concentric rings of heater cable at 1 and 2 m in diameter were installed 5 cm below the soil surface in heated plots. Unheated cables were installed similarly in control plots. Heating throughout the plot volume was generally even, ranging from 3.5 to 4.5°C except at 5 cm depth where the heated plots were on average only 2.4 ± 1.2°C warmer than the control due to a lack of aboveground heating. Soil moisture was slightly decreased in the warmed plots by an average of 1.5-3.5% volumetric water content (Hicks Pries et al., 2017).

2.2 Sample Collection and Analysis

Dataloggers (CR1000, Campbell Scientific, Utah, USA) continuously recorded soil temperature and moisture at 30 min intervals. Temperature was monitored at 5, 15, 30, 50, 75, and 100 cm depths at a radial distance of 0.75 m from the center of each plot. Temperature probes consisted of thermistors (Omega 44005) epoxied to PVC rods, placed inside thin-walled steel conduit. To monitor soil moisture, we used an enviroSCAN (Sentek, Australia) probe fitted with capacitance sensors at 10, 30, 50, and 90 cm at a radial distance of 0.75 m from the center of each plot. We calibrated the soil moisture measurements by comparing the sensor values at each depth to the volumetric water content measured in nearby (within 0.5 m) soil cores that were sampled five times over two years.

125 Each of the six plots has a set of gas wells at 15, 30, 50, 75, and 90 cm. The gas wells were 6.35 mm diameter stainless steel
126 tubes inserted into the soil at a 45° angle to the desired depth and topped with straight swage pipefittings (Swagelok Ohio,
127 USA) with septa. For CO_2 and $\delta^{13}CO_2$ measurements, samples were collected from the wells with a syringe on a nearly monthly
128 basis from March 2014 through June 2017 (32 months total) and always during morning hours. After clearing the headspace
129 in each well, a 25 ml gas sample was transferred to an evacuated 20 ml septum-topped glass vial. For analysis, 5 ml samples
130 were injected into the small sample isotope module of a cavity ring down spectrometer (CRDS, Picarro, Santa Clara,
131 California) where they were diluted with ultra zero air (without CO_2). A four-point calibration curve ranging from 2,000 to
132 20,000 ppm ($\delta^{13}C$=-26.7‰) was used to calculate the CO_2 concentration from the CRDS data and to correct for mass
133 dependency of the $\delta^{13}C$ measurement.
134
135 In July 2015, February 2016, April 2016, August 2016, March 2017, and June 2017, we collected additional samples from the
136 30 (except July 2015) and 90 cm gas wells into 13 ml flasks equipped with O-ring valves (LouwersHanique, Hapert,
137 Netherlands) to simultaneously measure CO_2 and O_2 concentrations in order to calculate ARQ. The flasks were analyzed in
138 the laboratory at the Hebrew University by a closed system (The Hampadah; Hilman and Angert, 2016). This fully automated
139 system uses an infra-red gas analyzer (IRGA) for CO_2 measurement (LI 840A LI-COR; Lincoln, NE, USA) and a fuel-cell
140 based analyzer (FC-10; Sable Systems International, Las Vegas, NV, USA) for measuring O_2. The flasks were analyzed within
141 2-3 weeks of collection.
142
143 In June 2017, we also ran a set of short (3 hour) incubations of root-free soil and of excised roots collected adjacent to the
144 experimental plots. We collected four mineral soil cores with a 5 cm diameter hammer corer, separated the cores into 0-20 and
145 20-40 cm depths, and removed roots >1 mm diameter. Roots were collected from four 25 cm x 25 cm x 25 cm soil pits. We
146 rinsed roots with water to remove soil and blotted them dry before placing them into mason jars. The root-free soil was also
147 placed into mason jars, and both sets of mason jars were flushed with ambient, outside air. After a three-hour incubation of the
148 root samples and a 21-hour incubation of the soil samples, the headspace was sampled for CO_2 and O_2 and analyzed as
149 described above. Incubations were run at room temperature, which was similar to the field temperature at the time of collection.

150 **2.3 Sample Calculations and Statistics**

151 To calculate ARQ, we used the following equation from Angert et al. (2015):

$$ARQ = -0.76\frac{\Delta CO_2}{\Delta O_2}$$

153 Where ARQ is the apparent respiratory quotient, ΔCO_2 (ppmv) is the difference between CO_2 concentrations in the soil pore
154 space gas and ambient (i.e., 0.5 to 1m aboveground) samples, ΔO_2 (ppmv) is the difference of the soil pore space O_2
155 concentration and ambient O_2 concentration, and 0.76 is the ratio of CO_2 to O_2 diffusivity in air (Massman, 1998). The negative
156 sign is for convenience so the ARQ value will typically be positive, because the difference in O_2 concentration is always

negative. For the jar incubations we used the same equation without the 0.76 factor. Ambient CO_2 concentrations were measured in the field at the time of sampling with the CRDS, while the ambient O_2 concentration was assumed to be 20.95% (Rumble, 2019). To relate the $\delta^{13}C$ value of soil pore space CO_2 to the $\delta^{13}C$ of CO_2 production, we corrected the pore-space $\delta^{13}C$ value for diffusion since ^{13}C diffuses slower in air than ^{12}C and thus the measured value does not accurately represent the value of production. For the correction, we used the following equation from Bowling et al. (2015):

$$\delta_{production} = \frac{C_s(\delta_s - 4.4) - C_a(\delta_a - 4.4)}{1.0044(C_s - C_a)}$$

Where C_s is the soil pore space CO_2 concentration (ppmv), δ_s (‰) is the isotopic composition of soil pore space CO_2 and C_a and δ_a are the CO_2 concentration and isotopic composition of ambient air, respectively. The ambient CO_2 concentrations and $\delta^{13}C$ values needed for these corrections were measured in the field at the time of sampling with the CRDS.

To investigate the effects of season, warming treatment, and soil depth on ARQ and $\delta^{13}C$, we ran multiple regressions in R (R Development Core Team, 2017). Because ARQ was not sampled from both depths on all dates, we ran separate regressions for each depth (30 and 90 cm) and then ran a regression that included a depth effect while dropping the first sampling date. In all regressions, treatment and sampling date (as a factor) were fixed effects. Following Zuur et al. (2009), we used a full model with all fixed effects and their interactions to optimize the random effects and autocorrelation structure based on AIC. For both versions, we used the individual gas well as a random effect and a temporal autocorrelation did not improve the model, nor did an autocorrelation function graph indicate one was needed. We chose the significant fixed effects by performing a series of pairwise model comparisons using AIC and the F test, dropping the least significant variables each time until only variables that improved the model fit remained. The p-values reported are those from the t-tests of the summary.lme function of best fit model. We report conditional R^2 values calculated using the rsquared command in the piecewiseSEM package.

To investigate seasonal patterns in $\delta^{13}CO_2$, we had more data in terms of both length of time and temporal density of sampling and were thus able to treat month as a continuous variable. We fit a sine function and tested models including the first and second harmonics of the month effect as well as linear fixed effects of depth, treatment, and a depth by treatment interaction. Graphical exploration indicated the sinusoidal pattern differed slightly by year, so we also added a year effect to the second harmonic of the month effect. As above, we used the full fixed effect model to test the best random and autocorrelation structure. Individual gas well depth was used as a random effect and a correlation structure did not improve the model.

To test relationships between ARQ and $\delta^{13}CO_2$, and both ARQ and $\delta^{13}CO_2$ individually versus soil temperature and volumetric water content, we ran mixed-model regressions with individual gas well as a random effect. For the soil climate relationships, we used AIC and the F test to investigate whether the warming treatment and its interaction with soil temperature or VWC were significant fixed effects. We tested the need for autocorrelation structures based off of AIC and none improved the models. For all models, we graphically checked the residuals for violations of normality and heterogeneity of variance. For

190 $\delta^{13}CO_2$ analyses, we dropped the 15 cm depths due to their unusually low $\delta^{13}C$ value (<-32‰) after correction (Eq. 1), which

191 indicated potential intrusion of atmospheric air during sampling that led to an overcorrection. We used one-way ANOVA's to

192 compare the ARQ of soil and root incubations and the ARQ of two soil depths we incubated. All statistics were performed in

193 R v 3.4.1 and regressions were done using the lme function (R Development Core Team, 2017).

3 Results

195 Both ARQ and $\delta^{13}CO_2$ had similar, strong seasonal patterns (Fig. 1a and 1b). ARQ values were higher during the growing

196 season (0.89 ± 0.01, n=42) and lower during the winter (0.70 ± 0.02, n=23). In ARQ regression analyses for both depths, there

197 was a significant effect of date (p<0.0001, n=59) with February 2016 and March 2017 differing significantly from July 2015

198 (90 cm only), April 2016, August 2016, and June 2017. Similarly, $\delta^{13}C$ was higher during the summer (June through October,

199 -27.97 ± 0.06, n=311) and lower during the winter and spring (November through May, -29.01 ± 0.04, n=447). While individual

200 dates were not compared statistically for $\delta^{13}CO_2$, the vast improvement in model fit using month as a sine function instead of

201 a linear function or factor (ΔAIC=114) is strong statistical evidence for a seasonal effect (Fig. 2b). ARQ and $\delta^{13}CO_2$ were

202 significantly related according to the mixed effect regression model (Fig. 1c, p<0.0001, n=64, R^2=0.20). However, the patterns

203 in ARQ and $\delta^{13}CO_2$ did not match during April.

204

205 Both ARQ and $\delta^{13}CO_2$ differed by warming treatment (Fig. 2) and by depth (Table 2). For the ARQ of 30 cm depths, there

206 was a significant treatment-by-date interaction (p=0.051, n=30) whereby heated plots had greater ARQ values during the

207 winter months (February 2016 and March 2017; Fig. 2a). For the ARQ of 90 cm depths, the best fit model did not include a

208 significant treatment effect or treatment-by-date interaction (Fig. 2a, n=35). For $\delta^{13}CO_2$ across all depths, treatment was a

209 significant effect (p=0.0065, n=758) with warmed soil on average having a slightly higher $\delta^{13}CO_2$ (-28.33 ± 0.05) than the

210 control soil (-28.83 ± 0.06; Fig 2b). The treatment-by-depth interaction was not significant for $\delta^{13}CO_2$ and was not included in

211 the best fit model. Looking at depth only (Table 2), ARQ at 30 cm was marginally significantly greater than ARQ at 90 cm by

212 0.07 units (p=0.099, n=59), while $\delta^{13}CO_2$ increased with depth from -28.98 at 30 cm to -28.34 at 90 cm (p=0.0089, n=758).

213

214 Both ARQ and $\delta^{13}CO_2$ showed strong relationships with soil climate that were significantly affected by the warming treatment

215 (Fig 3). We tested relationships with soil temperature and soil moisture individually because of the strong negative correlation

216 between temperature and moisture in this Mediterranean climate (pearson's r=-0.76 to -0.78). ARQ increased significantly

217 with increasing soil temperatures (p<0.0001, n=65, R^2=0.52; Fig 3a) with values increasing faster in control plots than in

218 warmed plots (p=0.0051). ARQ decreased with increased soil moisture (p<0.0001, n=60 due to missing VWC values, R^2=0.24;

219 Fig. 3b), and the decrease was faster in the control than in the warmed plots. $\delta^{13}CO_2$ became higher with increasing soil

220 temperatures (p<0.0001, n=375, R^2=0.33; Fig. 3c) with values again increasing faster in the control than in the warmed plots

221 (p=0.02). $\delta^{13}CO_2$ decreased with increased soil moisture (p<0.0001, n=345 due to missing VWC values, R^2=0.30; Fig. 3d),

222 and treatment did not have a significant effect.

223

224 Our incubations of roots (n=4) and of root-free soil (n=4 per depth increment) indicated that heterotrophic and autotrophic

225 respiration had significantly different ARQ values, at least during the summer when we performed the incubations. Roots had

226 a greater ARQ (0.87 ± 0.03) than did root-free soil (0.78 ± 0.02; one-way ANOVA, p=0.029). Furthermore, ARQ of the soil

227 incubations significantly declined with depth from 0.82 ± 0.01 at 0-20 cm to 0.74 ± 0.02 at 20-40 cm (one-way ANOVA,

228 p=0.0053).

229 **4 Discussion**

230 There are many factors that can affect ARQ; however, our evidence indicates the strong seasonal patterns in ARQ and $\delta^{13}CO_2$

231 were likely driven by changes in the amount of root-derived organic substrates providing energy for heterotrophic microbial

232 respiration and changes in the contributions of autotrophic root respiration. This interpretation is supported by previous soil

233 ARQ studies, our incubations, and the scientific understanding of how plant carbon inputs change seasonally. The seasonal

234 range in ARQ from ≈0.9 during the growing season to ≈0.7 during the winter may reflect a shift in the molecules fueling

235 respiration from more oxidized substrates like sugars and organic acids derived from roots in the summer to more reduced

236 substrates in the winter such as lipids and proteins derived from microbial necromass. Previous incubations found that glucose

237 additions increased ARQ (Dilly, 2001; Theenhaus et al., 1997). Other studies attributed a decline in ARQ during the time

238 course of incubation to the depletion of labile carbon sources (Angert et al., 2015; Severinghaus, 1995). Our short-term

239 incubations demonstrated that root respiration has a greater ARQ than microbial respiration from root-free soils. During the

240 growing season, root respiration and exudation increase, which should increase ARQ, as seen in our data. In Eastern U.S.

241 deciduous forests, root exudation rates tend to be lower in the winter and spring than in the summer and fall (Abramoff and

242 Finzi, 2016; Phillips et al., 2008). Mass-specific fine root respiration rates were greater during the growing season (up to 8

243 nmol CO_2 g^{-1} s^{-1}) than in the winter (<1 nmol CO_2 g^{-1} s^{-1}) and total belowground carbon flux was greatest from May through

244 October (Abramoff and Finzi, 2016). Though these root studies were not from the western United States, eddy covariance data

245 from a coniferous forest near our study site found that primary production was greatest during the summer months from June

246 through mid-September (Goldstein et al., 2000).

247

248 Beyond the results of our root and root-free soil incubations, there is additional evidence that root and rhizosphere respiration

249 should have a greater ARQ than microbial-derived respiration. For example, respiration of root tips is driven by sugar content

250 and has an RQ of 1.0 (Saglio and Pradet, 1980). Furthermore, recent metabolomic analysis of root exudates identified sugars,

251 carboxylic acids, amino acids, and phenolics as the main metabolites (Zhalnina et al., 2018), most of which are relatively

252 oxidized energy sources with relatively high respiratory quotients. Thus, we would expect greater ARQ values during the

253 summer due to higher root activity. When trees are dormant, the lack of fresh inputs from roots may lead to more recycling of

254 organic carbon within microbial biomass, wherein proteins and lipids are the first and third largest constituents by weight,

255 making up to 55% and from 10-35% of a typical bacterial cell's dry mass, respectively (Kleber and Reardon, 2017; Neidhardt,

256 1987). Lipids and proteins tend to be reduced and have the lowest RQ values of common organic substrates, likely explaining

257 the lower wintertime ARQ values in our soils.

258

259 The seasonal pattern in $\delta^{13}CO_2$ reinforces our interpretation that changes in respiration carbon sources were driving changes

260 in ARQ. Soil $\delta^{13}CO_2$ was more enriched in the summer and became more depleted in the winter by up to 2‰. In a

261 comprehensive review of carbon isotopes in terrestrial ecosystems, Bowling et al. (2008) showed that plant lipids tend to be

262 more depleted in ^{13}C while sugars and organic acids tend to be more enriched in ^{13}C relative to bulk leaf $\delta^{13}C$. While these

263 numbers are based on plant lipids, if we assume microbial lipids are similarly depleted relative to other organic compounds,

264 an increase in microbial necromass as an organic matter source relative to root-derived sources during the winter would cause

265 the observed fluctuation in $\delta^{13}CO_2$. Furthermore, a chemical fractionation of soil organic matter found that the water-soluble

266 fraction, which includes sugars, was 3-4‰ more enriched than the acid-insoluble pool (Biasi et al., 2005). While the

267 interpretation of respiration $\delta^{13}C$ by itself in C_3 ecosystems can be difficult due to the small ‰ differences among carbon

268 sources (e.g., Bowling et al., 2015), the simultaneous use of ARQ and $^{13}CO_2$ helps strengthen interpretations.

269

270 Seasonality encompasses changes to phenology and soil climate, among other factors. Both ARQ and $\delta^{13}C$ had significant

271 positive relationships with soil temperature. In addition to the importance of plant phenology described above, temperature

272 could have direct effects on respiration sources. Specifically, warmer temperatures can increase root exudation rates (Yin et

273 al., 2013) and the relative contribution of autotrophic-derived, if not directly autotrophic, respiration to total soil respiration.

274 In two subarctic ecosystems, warming increased the proportion of ecosystem respiration derived from autotrophs (which, using

275 natural abundance radiocarbon as a tracer, included heterotrophic respiration of root exudates) relative to heterotrophs (Hicks

276 Pries et al., 2015). However, temperatures can affect ARQ through more than just changing the contributions of autotrophic

277 sources. Lower temperatures increase the thermodynamic favorability of the oxidation of reduced carbon in compounds like

278 lipids (LaRowe and Van Cappellen, 2011), which could also explain the decrease in ARQ values at lower temperatures. For

279 $\delta^{13}C$, it is likely that phenological changes to organic carbon sources were more important than temperature per se. Several

280 soil incubation studies show that increases in temperature cause respired $\delta^{13}CO_2$ to decrease by about 0.12–0.35‰ for each

281 1°C rise in temperature—the opposite of the relationship we found (Andrews et al., 2000; Biasi et al., 2005; Hicks Pries et al.,

282 2013). In these incubations, which were devoid of new organic carbon inputs, unlike in situ conditions, the shift was attributed

283 to changes to the microbial community that affected carbon source preferences (Andrews et al., 2000; Biasi et al., 2005).

284 Furthermore, in a Mediterranean climate, phloem sap from trees has been shown to become more enriched in ^{13}C during the

285 summer (Merchant et al., 2010), matching our pattern in soil $\delta^{13}CO_2$.

286

287 While ARQ and $\delta^{13}CO_2$ increased with soil temperature, experimental warming slowed that rate of increase so that both ARQ
288 and $\delta^{13}CO_2$ values were generally greater in the control than in the heated treatment at the warmest soil temperature.
289 Concurrently, during the colder months, experimental warming caused greater ARQ values (as at 30 cm depths in February
290 2016 and March 2017) and slightly higher $\delta^{13}CO_2$ relative to the controls. The increase in ARQ and $\delta^{13}CO_2$ with experimental
291 warming during the colder soil temperatures of winter indicates proportionately more respiration of relatively oxidized, labile
292 organic substrates in the heated treatment. Perhaps enhanced root growth and exudation in the heated treatment (Yin et al.,
293 2013) could result in the increased availability of labile organic substrates, but this increase occurred in winter when trees were
294 less active and was not seen during the growing season. The increase in ARQ and $\delta^{13}CO_2$ could also be the result of preferential
295 decomposition of more highly oxidized, labile substrates by a more active microbial population during the winter.
296 Experimental warming increased microbial activity at all soil depths; warming increased CO_2 production by 34 to 37% overall
297 with about 40% of the warming response occurring below 15 cm in the soil profile (Hicks Pries et al., 2017). A warming-
298 induced increase in the consumption of labile substrates could lead to exhaustion of the labile pool and eventually smaller
299 warming-induced SOC losses as seen at Harvard Forest (Melillo et al., 2002, 2017). In fact, the trend towards decreased ARQ
300 and $\delta^{13}CO_2$ values during the warmer soil temperatures of the growing season could be due to a depletion of the labile SOC
301 pool during the winter. Another potential explanation for lower values during the growing season could be a reduction in the
302 proportion of soil respiration derived from roots. In one warming study, root respiration was less sensitivity to warming relative
303 to heterotrophic respiration (Hartley et al., 2007). The warming treatment dried the soil slightly at Blodgett (Hicks Pries et al.,
304 2017), which could stress roots during California's essentially rainless growing season. Future measurements of CO_2
305 production, ARQ, and $\delta^{13}CO_2$ in trenched and untrenched plots could help distinguish these possibilities.
306
307 Soil temperature and soil moisture were so strongly negatively correlated due to our study site's Mediterranean climate that it
308 is difficult to separate their effects. ARQ and $\delta^{13}CO_2$ were negatively correlated with volumetric water content, which was
309 greatest when soil temperatures were coldest. Volumetric water content has the potential to control ARQ in several ways. First,
310 increased soil moisture reduces O_2 availability, which could increase ARQ values >1 as CO_2 is produced without O_2
311 consumption. However, during our study the soil remained oxic (soil O_2 averaged 20% and the minimum was 17.38%). The
312 negative relationship between ARQ and soil moisture indicates that anaerobic respiration was not a driver, and we only
313 measured one ARQ value greater than one (1.03) during our study. However, diffusion rates are lower with higher soil
314 moisture, which could make detection of high ARQ values difficult if anoxic conditions occur within microaggregates. In
315 anoxic microaggregates, iron (II) is produced anaerobically, which is subsequently oxidized to iron (III) as the aggregate dries
316 and becomes aerobic, a process that consumes O_2 without producing CO_2, resulting in low ARQ values that can be detected
317 as drying soils increase diffusion (Angert et al., 2015). In our soils, which tend to contain relatively high amounts of iron
318 oxides (Rasmussen et al., 2005), iron oxidation could explain the 15% of ARQ values that were less than the reduced organic
319 matter value of 0.7. Lastly, since CO_2 is more soluble in water than is O_2, more CO_2 relative to O_2 is expected to dissolve in
320 soil water, which would reduce ARQ values at higher moisture contents. However, different dissolution rates and iron

oxidation do not fully explain our data as the wide variability in ARQ values (0.44 to 0.94) at high volumetric water contents (0.27 to 0.31) can be best explained by time of year (Fig. A1), which again points to phenology as the main driver; the greater ARQ values are from April and June while the lower values are from February and March. Furthermore, there was a stronger relationship between observed and predicted ARQ in the temperature model than in the soil moisture model.

Experimental warming affected the relationship between ARQ and soil moisture. ARQ was greater in the heated treatment when soil moisture was high (winter) and lower in the heated treatment when soil moisture was low (growing season). Soil water sampled from lysimeters had a greater concentration of dissolved organic carbon in the warming treatment than in the control (unpublished data), which could deliver oxidized substrates to microbes during the winter rainy season.

The reasons for $\delta^{13}CO_2$ decreasing with increasing volumetric water content are not clear. Based on kinetics, we would expect that as more CO_2 dissolves in water, the soil air should become enriched in $^{13}CO_2$ because dissolution discriminates against the heavy isotope and increasingly so at lower temperatures (Zhang et al., 1995), but our data were not consistent with this explanation. Another possibility is that advective transport of atmospheric CO_2 through the soil is more likely at lower soil moisture content. While intrusion of atmospheric CO_2 would increase the $\delta^{13}C$ of soil air, it reduces the effective diffusion fractionation to <4.4 ‰, leading to overcorrected, and thus unrealistically low $\delta^{13}C$ values, of which we did have several.

Depth was the only parameter by which ARQ and $\delta^{13}CO_2$ did not change in concert with one another. ARQ decreased with depth while $\delta^{13}CO_2$ increased. The decrease in ARQ with depth, which was more dramatic in the root-free soil incubations than in soil air (difference of 0.08 versus 0.03), is likely due to decreased plant inputs with fewer fine roots and less root exudation at depth (Hicks Pries et al., 2018; Tückmantel et al., 2017). The enrichment of soil $\delta^{13}CO_2$ likely reflects the near-universal enrichment of soil organic carbon with depth due to catabolic carboxylation reactions (as microbial byproducts and necromass become a larger proportion of soil organic matter; Ehleringer et al., 2000; Torn et al., 2002) or the Suess effect (the continuing depletion of atmospheric CO_2 over time due to the burning of fossil fuels). In our soils, there was about a 2‰ enrichment in bulk soil organic $\delta^{13}C$ with depth (Hicks Pries et al., 2018).

5 Conclusion

Here we have shown, for the first time, both annual patterns in soil ARQ and how ARQ is affected by experimental warming. We inferred that seasonal patterns in ARQ were likely due to changes in the dominant substrates providing the energy for soil respiration with root-derived sugars and organic acids being the dominant substrates during the growing season and microbial necromass being the dominant substrate during the winter. These inferences of organic substrates were supported by soil $\delta^{13}CO_2$ measurements, which showed clear patterns despite our study system containing only C_3 plants. We caution that direct experimental evidence of how ARQ changes with sources is needed before our inferences of substrate use can be proven.

However, our data indicate ARQ measurements can help to disentangle the biological sources contributing to soil respiration and to understand how sources are shifting due to global change. This application of ARQ worked well in our soils, which were well-drained, oxygenated, and lacked carbonates. The interpretation of soil ARQ values becomes more complex if those conditions are not met (Angert et al., 2015). The autotrophic and heterotrophic source separation in our incubations indicates ARQ has the potential to be used to partition soil respiration in a similar manner to natural abundance δ^{13}C (e.g., Dorrepaal et al., 2009; Hicks Pries et al., 2013). To enable further applications of ARQ, more characterization is needed of the controls of the ratio, including incubation studies of sterile and 'live' soils under aerobic and anaerobic conditions and co-located measurements of ARQ fluxes and the oxidative ratio of organic matter sources as in Masiello et al. (2008). Such future investigations will help determine whether ARQ deserves a prominent place alongside natural abundance isotopes in the ecosystem ecology and biogeochemistry toolkit.

Code and Data Availability

Data (doi:10.15485/1596312) are publicly available on ESS-DIVE (http://ess-dive.lbl.gov/). The R code used for the statistics and to generate the figures in this paper is available as a supplement.

Author Contribution

CHP, AA, and MST conceived of the study. Field measurements were conducted by CHP and CC. Lab analyses were conducted by CHP and BH. Statistical analyses were conducted by CHP. CHP wrote the manuscript with feedback from all authors.

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Molecule	RQ[a]	$\delta^{13}C$ (relative to bulk leaf)[b]
Organic acids	1.4 (0.97-4.2)	+0.75
Sugars	1.0	+1.5-2
Phenolics	0.95 (0.92-1.3)	NA
Proteins	0.77 (0.67-1.01)	+1
Lignin	0.88 (0.88-0.94)	-3
Lipids	0.73 (0.68-0.80)	-4

510 [a] Data from Masiello et al. 2008
511 [b] Data from Bowling et al. 2008

512 **Table 1. Respiration quotients (RQ; the inverse of reported oxidative ratios, which are based on elemental analyses) and relative**
513 **isotopic enrichment of common molecules/substrates for respiration found in soils. The most common RQ value is listed followed by**
514 **the range of potential RQ values in parentheses. The apparent respiration quotient is based on the simultaneous measurement of**
515 **soil CO_2 and O_2.**

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Depth (cm)	$\delta^{13}CO_2$ (‰)	ARQ
30	-29.0 ± 0.09 (191)	0.84 ± 0.02 (30)
50	-28.6 ± 0.08 (190)	
70	-28.4 ± 0.07 (191)	
90	-28.3 ± 0.08 (186)	0.81 ± 0.02 (35)

521 **Table 2. The mean ± SE (number of samples) of corrected $\delta^{13}CO_2$ and ARQ of soil pore space by depth averaged over all timepoints.**

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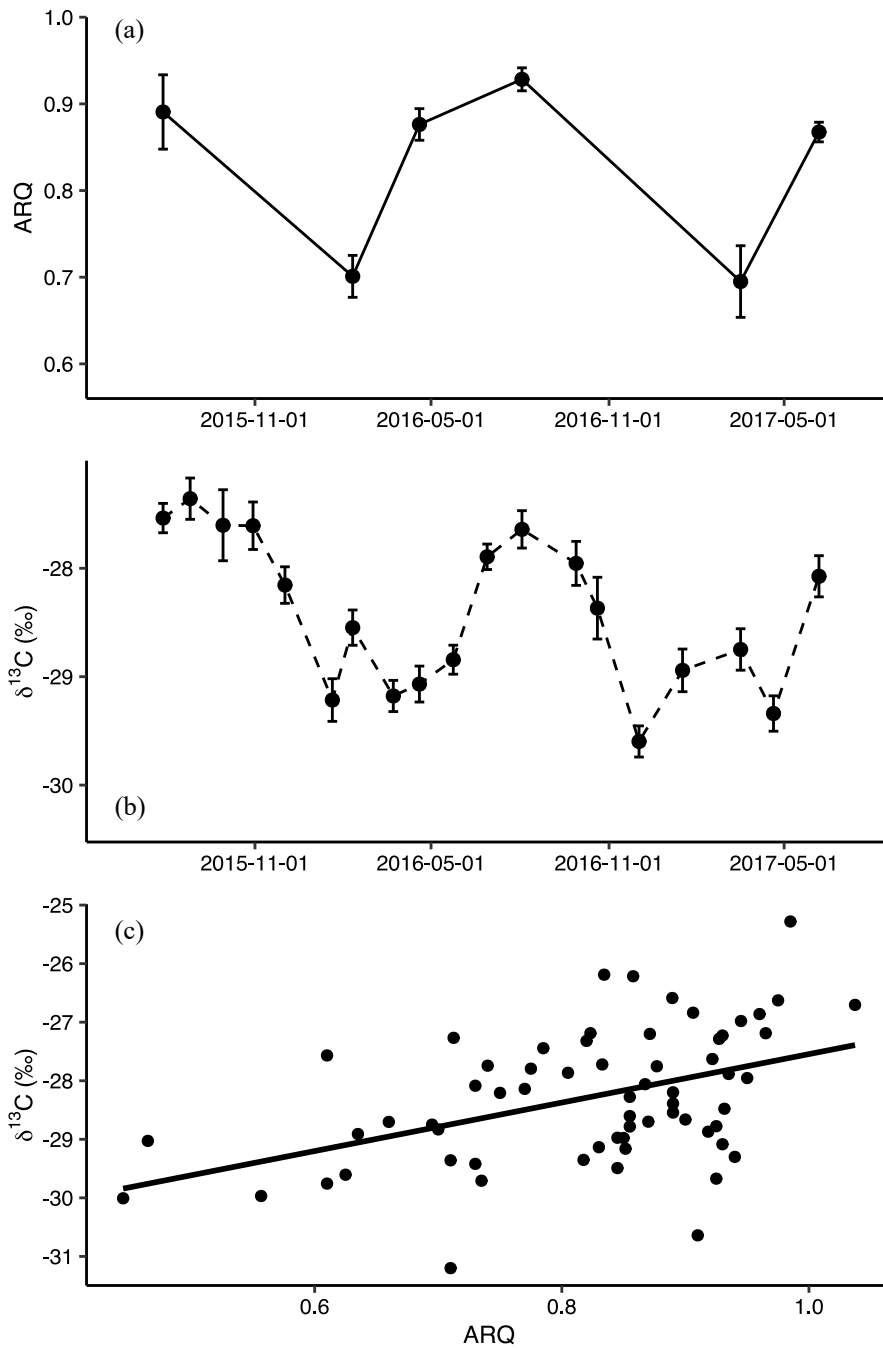
523

524 **Figure 1. Mean (± SE) apparent respiration quotient (ARQ, n=12 except n=6 for 07/2015; a) and corrected δ¹³CO₂ (n=24 per date;**
525 **b) in soil pore air averaged across all depths and treatments by sampling month. The relationship between ARQ and δ¹³CO₂ values**
526 **over the months when they were sampled simultaneously (c). The line shows the fit of a linear regression (p<0.0001, n=64, R²=0.20).**

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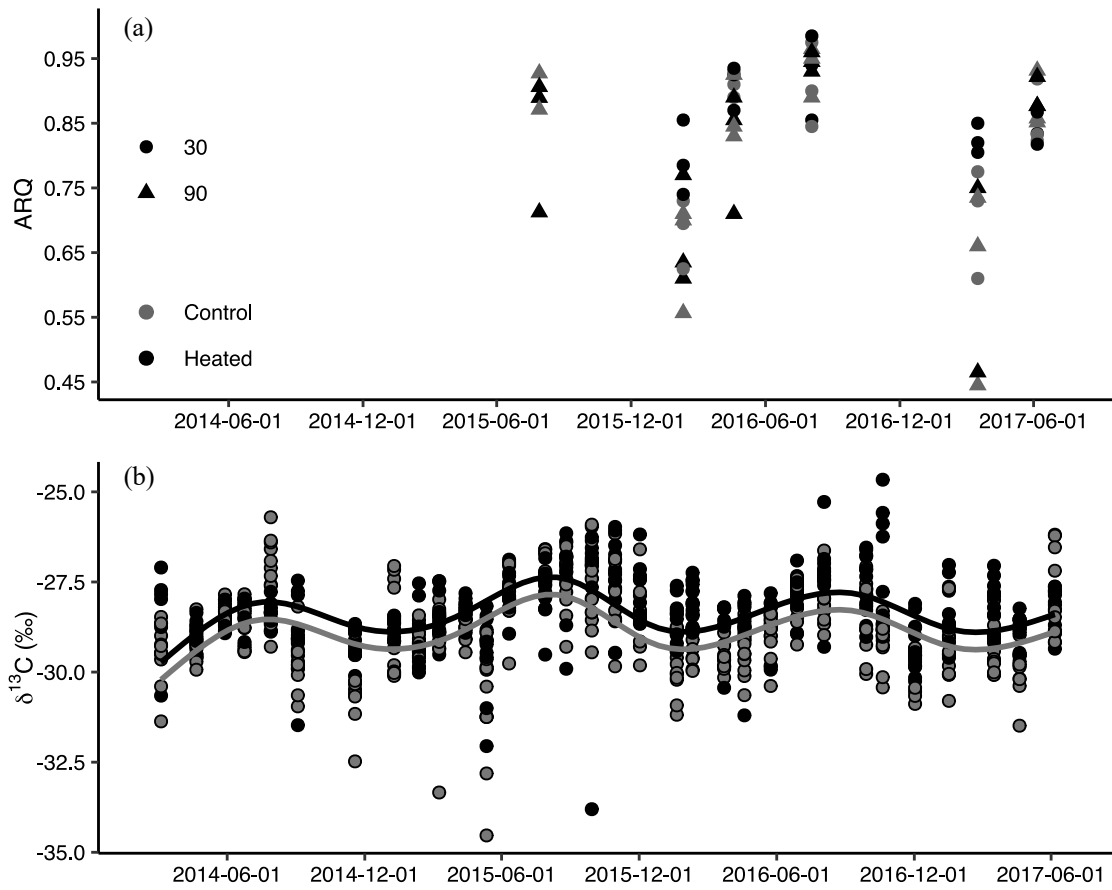
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529 **Figure 2. Apparent respiration quotient (ARQ) by sampling date for heated (black) and control (grey) treatments at 30 cm (circles)**
530 **and 90 cm (triangles) depths (n=3 per date and depth combination; a). ARQ differed significantly among treatments during the**
531 **winter at 30 cm. Corrected δ^{13}CO$_2$ for all depths (30, 50, 70, and 90 cm) and months sampled (b). The lines represent the predicted**
532 **fit of a sinusoidal regression (see text) for an average soil depth in control (grey) and heated (black) treatments (n=758).**

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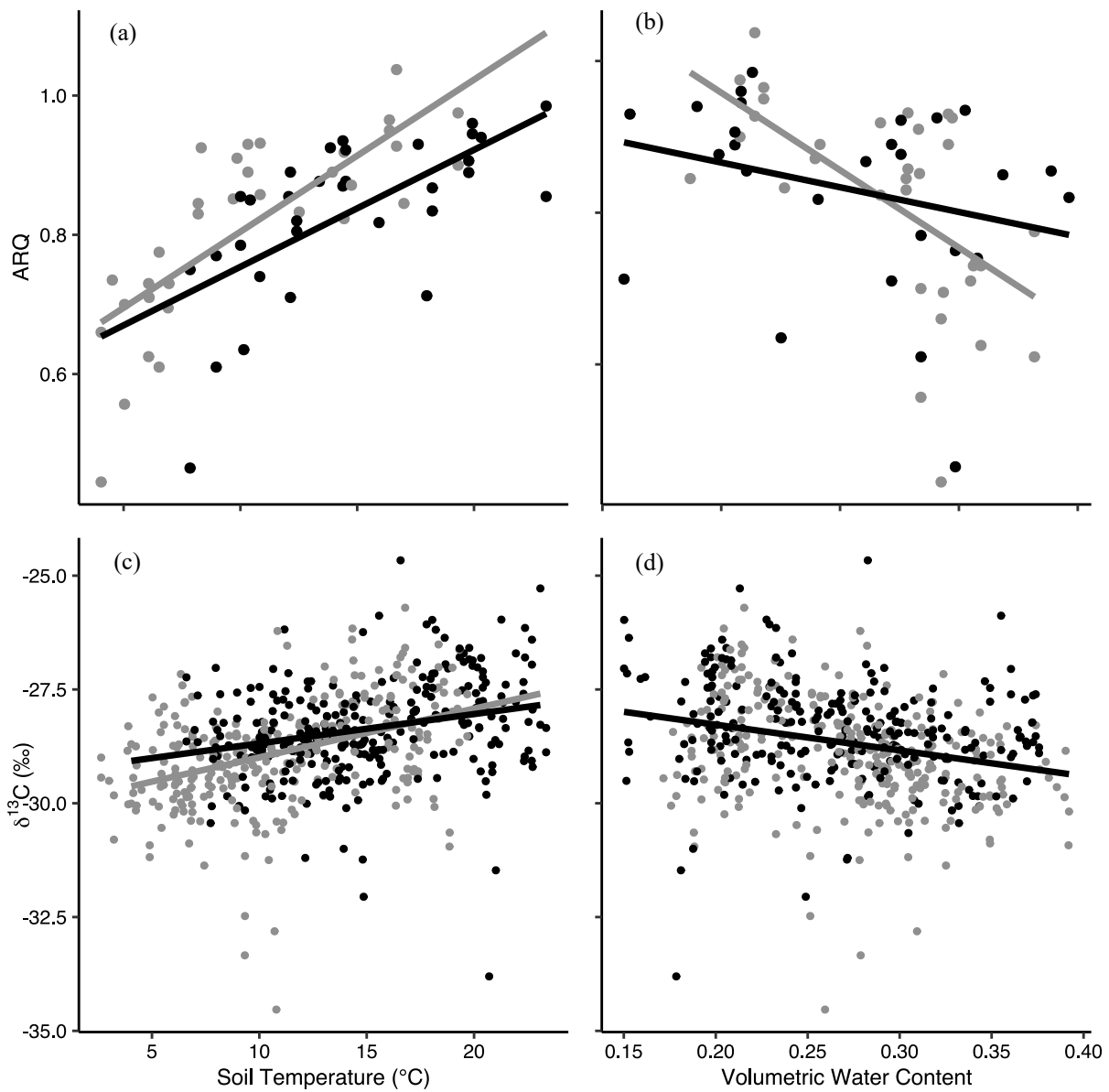
Figure 3. The relationships of apparent respiration quotient (ARQ) by soil temperature (a, n=65) and soil moisture (b, n=60) and $\delta^{13}CO_2$ by soil temperature (n=565, c) and soil moisture (n=535, d). Gray and black points represent data from control and heated gas wells, respectively. The lines show the fit of a mixed model regression between each variable where individual gas well was treated as a random effect. Separate grey (control) and black (heated) lines indicate there was a significant effect of warming treatment on the relationship between the response variable and soil temperature.

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