Variability of the Apparent Respiratory Quotient of a forest Soils and Tree Stems

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Key Points:

- The ratio of CO_2/O_2 fluxes in respiration (ARQ) depends on the substrate stoichiometry and on additional processes reacting with the gases
- Bulk-soil ARQ value is governed by stoichiometry in high respiration rates and by processes like abiotic Fe^{2+} oxidation in low rates
- ARQ can be used to partition soil respiration sources as shown by distinct bulk-soil and roots ARQ values that confine total soil value

Abstract

The CO_2/O_2 fluxes ratio (ARQ) measured in soils and plants contains valuable information about the respiratory-substrate stoichiometry and biotic and abiotic non-respiratory processes reacting with the gases. For meaningful use in biogeochemical studies it is necessary to resolve the substrate and processes effects. In addition, unique ARQ signatures can be used to weight contributions to soil respiration. We investigated these uses by measurements in soil pore space air (ARQ_{sa}), and in headspace air from incubations of bulk-soil (ARQ_{bs}) and tree stem-tissues (ARQ_{ts} for fresh tissues; ARQ_{ts24} after 24-h storage) in 10 measurement campaigns over 15 months in a Mediterranean oak forest. Mean (range) values were: ARQ_{sa} = 0.76 (0.60-0.92), ARQ_{bs} = 0.75 (0.53-0.90), ARQ_{ts} = 0.39 (0.19-0.70), and ARQ_{ts24} = 0.68 (0.42-1.08). Both ARQ_{ts} and ARQ_{ts24} were below 1.0, the value expected for carbohydrate respiration in plants. Involvement of non-respiratory processes like non-phototrophic CO_2 re-fixation and wound-response O_2 uptake (for ARQ_{ts}) can explain the results. The mean ARQ_{bs} (0.75) probably represents the stoichiometry of the respiratory substrate, which is lower than expected using bulk soil organic matter (SOM) stoichiometry (~0.95), suggesting a labile, less oxidized, SOM pool contributes more to respiration fluxes. Abiotic O_2 uptake by Fe^{2+} was demonstrated to reduce ARQ_{bs} to 0.37, at the most, but estimated to have small effect under typical respiration rates. ARQ_{sa} was usually higher than ARQ_{bs} and lower than root ARQ (which, when measured, ranged from 0.73-0.96), demonstrating the potential of ARQ to partition the autotrophic and heterotrophic sources of soil respiration.

Plain Language Summary

Carbon dioxide is produced by the processes of both metabolic respiration by plants and microbial respiration and decomposition in soils. These are among the most important processes in terrestrial ecosystems, both oxidizing organic compounds using O_2 and emitting the resulting CO_2 to the atmosphere. However, our understanding of these processes, which are often collectively referred to as "respiration" is still incomplete. Here we investigated the use of the measured ratio CO_2 released to O_2 consumed, termed the apparent respiration quotient (ARQ), to investigate respiration in soils and tree stems. ARQ measurements are rarely made, but can provide valuable information about the chemistry of the respiratory substrates, and about additional processes that involve CO_2 and O_2 cycling in terrestrial ecosystems. The expected substrates (carbohydrates) in tree stems and soils yield ARQ ≈1; however, we measured considerably lower values. The low ARQ values in the soil can be explained if microbes decompose compounds with low amounts of oxygen, which is surprising. No substrates can produce ARQ values as low as those we measured in stem core incubations, indicating another processes at work.

1 Introduction

New measurement methods can improve the understating and prediction of ecosystem processes. An emerging tool in biogeochemical studies is the coupled measurement of CO_2 and O_2 fluxes. In respiration the ratio CO_2 production/O_2 consumption is termed the 'respiratory quotient' (RQ). The inverse term 'oxidative ratio' (OR, 1/RQ) is also used, especially when describing ecosystem processes that include photosynthesis with O_2 production (where the fluxes direction is opposite to respiration). Both ratios depend primarily on the stoichiometry, of the respiratory substrate or the net synthesized biomass. The stoichiometry of organic molecule determines the mean oxidation state (C_{ox}) of the C atoms in the molecule [*LaRowe and Van Cappellen*, 2011; *Masiello et al.*, 2008]. The more oxidized (higher C_{ox}) the molecule, the fewer moles of O_2 are consumed per mole of CO_2 released during complete oxidation and the RQ is higher (Table 1). For this reason RQ is often used to infer which respiratory substrate is being used by plants and animals. However, in soils and isolated plant organs additional biotic and abiotic non-respiratory processes can influence CO_2 and/or O_2 and lead to a ratio of CO_2/O_2 that differs from the change the substrate's RQ value. For this reason, we refer to the ratio CO_2 efflux/O_2 uptake measured in tree stems and soils as the 'apparent RQ' (ARQ) [*Angert and Sherer*, 2011; *Angert et al.*, 2015]. The main processes with the potential to affect ARQ are presented in Figure 1 and detailed in section 1.1. In addition, in a recent study we suggested that ARQ measurements could be used to separate autotrophic and heterotrophic respiration sources in soils due to different ARQ signatures [*Hicks Pries et al.*, 2019]. Thus, ARQ has the potential to become a useful tracer, similar to $\delta^{13}C$ in the ability to identify respiratory substrates, processes, and to separate respiration sources [*M E Gallagher et al.*, 2017].

For meaningful use of ARQ as a biogeochemical tracer, it is crucial to identify and separate the signals related to substrate stoichiometry and to non-respiratory processes. In addition, the sources of ARQ variability in soils and tree stems are still unclear. Addressing these questions has become more feasible with the development of user friendly O_2 sensors based on fuel-cell and optical quenching technologies. In the current study we explore ARQ variability and its sources in soils and tree stems of a Mediterranean oak forest, and discuss next research steps.

Table 1 – Selected Compounds and their Type, Formula, Mean Oxidation State of the C (C_{ox}), RQ, OR, and the Gibbs Energy.

Compound	Type	Formula	C_{ox} [a]	RQ (ARQ) [b]	OR [c]	Gibbs energy (kj mol C^{-1}) [d]
Oxalic acid	Organic acid	$C_2H_2O_4$	3	4	0.25	-25.2
Glucose	Carbohydrate	$C_6H_{12}O_6$	0	1	1	60.3
Cellulose	Structural polysaccharide	$(C_6H_{10}O_5)_n$	0	1	1	60.3
Aspartic acid	Amino acid	$C_4H_7NO_4$	1	1.33 (NH_3) 0.80 (HNO_3)	0.75 (NH_3) 1.25 (HNO_3)	31.8
Lignin [e]	Structural polymer	$C_{100}H_{124}O_{43}$	-0.38	0.91	1.10	71.13
Oleic acid	Fatty acid (lipid)	$C_{18}H_{34}O_2$	-1.66	0.71	1.42	107.8
Methane	Inorganic gas	CH_4	-4	0.5	2	174.3

[a] The mean oxidation state of C in the molecule, known also as NOSC (the average nominal oxidation state of carbon) calculated by $C_{ox} = \frac{2d-b+3c-z}{a}$ where the coefficients are according to the molecule formula $C_aH_bN_cO_d^z$ while z corresponds to the net charge [*LaRowe and Van Cappellen*, 2011; *Masiello et al.*, 2008]. This calculation assumes all non C species have fixed oxidation state during formation or break down. C_{ox} is related to RQ by $RQ = \frac{4}{4-C_{ox}}$. [b] The respiratory quotient (RQ), the ratio CO_2 efflux / O_2 uptake in full aerobic oxidation of the compound. We use the term ARQ (apparent RQ) since in soils and tree stems measurements additional processes can cause deviation from the theoretic RQ. When molecule contains N the RQ and OR values depend on the N oxidation state in the respective end and source molecules (e.g. NH_3 (-3) or HNO_3 (+5)). [c] The oxidative ratio (OR = 1/RQ). Often viewed as the ratio O_2 produced/ CO_2 assimilated required for the biosynthesis of the compound. [d] The Gibbs energy for oxidation half reactions at 25°C and 1 bar calculated using *LaRowe and Van Cappellen's* (2011) Eq. 14: $G^0_{Cox} = 60.3 - 28.5 * C_{ox}$. [e] Lignin formula is according to Baldock et al. 2004.

1.1 Observed ARQ in soil respiration components and tree stems

The ARQ of soil heterotrophic respiration is usually approximated by root-free bulk-soil incubations (ARQ_{bs}). According to meta-analysis of C_{ox} in soil organic matter (SOM) [*Worrall et al.*, 2013] ARQ_{bs} values are expected to range between 0.77 and 1.11 with median of 0.95. However, values of 0.27-0.94 measured previously from a variety of natural ecosystems and agricultural lands are mostly below these expected values [*Angert et al.*, 2015; *Aon et al.*, 2001a; b; *Dilly*, 2001; 2003; *Dilly and Zyakun*, 2008; *Severinghaus*, 1995]. Reasons for measured ARQ in incubations to differ from the values expected from C_{ox} of bulk soils include (Fig. 1): (1) processes involving electron transfer that affect either O_2 uptake or CO_2 production without affecting both gases; (2) interactions involving the greater solubility of CO_2 in water and inorganic C cycling that do not affect O_2 simultaneously; (3) other processes that affect CO_2

more than O_2 or vise versa such enzymatic uptake of CO_2 by 'dark fixation' that affect CO_2 more than O_2.

Enhanced O_2 uptake derived from abiotic oxidation of reduced species like Fe^{2+} and Mn^{2+} increases the denominator of the ARQ ratio and thus decreases its value. The opposite effect on ARQ is expected during anoxic conditions when oxidized Fe^{3+} and Mn^{3+} for example are used as an alternative electron acceptors. In that case, CO_2 is emitted without any O_2 uptake and the numerator of the ARQ ratio increases. Anoxic conditions may exist within soil aggregates even in aerated soils [*Druschel et al.*, 2008; *Hall and Silver*, 2013; *Sexstone et al.*, 1985], but become more important after soil wetting as diffusion in water is slower by orders of magnitude than diffusion in air, and when respiration rates are high and O_2 replenishment in microsites cannot meet respiratory needs.

Carbon dioxide reacts with water to form weak acids (HCO_3^- and CO_3^{2-}) and is ~30 times more soluble than O_2. Storage of respired CO_2 as dissolved inorganic carbon (DIC) in soil water therefore can also lower the measured ARQ_{bs}, especially in soils that may contain calcium carbonate. At higher soil pH the concentrations of HCO_3^- and CO_3^{2-} increase, and therefore also the capacity of the soil water to hold DIC. However, if the water containing the DIC does not leach, the CO_2 is expected to degas back to the soil pore space when the soil is dried. In calcareous soils mainly in arid and semi-arid regions large ARQ_{sa} deviations are expected due to precipitation and dissolution of carbonates [*Angert et al.*, 2015; *Benavente et al.*, 2010; *Cuezva et al.*, 2011; *Emmerich*, 2003; *T M Gallagher and Breecker*, 2020; *Ma et al.*, 2013].

Other processes involving CO_2 and O_2 include root uptake, SOM oxidation processes, and dark fixation. DIC (or CO_2) uptake by roots is probably not substantial due to anatomical features [*Ubierna et al.*, 2009]. 'Oxidative depolymerization' is the addition of oxygen to macromolecules that forms smaller and soluble molecules, which are more readily digested by microbes [*Kleber et al.*, 2015]. If the oxygen-rich molecules are not respired and rather bind to mineral surfaces, there is a net O_2 uptake and ARQ is lowered. Dark fixation of CO_2 by the microbial community is another process that can lower ARQ_{bs} [*Akinyede et al.*, 2020; *Miltner et al.*, 2005].

In plants, the primary respiration substrates are carbohydrates with ARQ = 1.0 [*Hoch et al.*, 2003; *Plaxton and Podestá*, 2007]. This value is expected for roots, but measurements of instantaneous ARQ from roots (ARQ_{root}) often deviate from 1.0, ranging from 0.79 -1.4 [*Hawkins et al.*, 1999; *Rachmilevitch et al.*, 2006; *Shane et al.*, 2004] (Fig. 1). These studies measured ARQ from roots (from intact plants or excised) submerged in nutrient solution. ARQ_{root} values greater than 1.0 were explained by nitrate assimilation that consumes electrons otherwise delivered to O_2 [*Bloom et al.*, 1989; *Lambers et al.*, 2008; *Rachmilevitch et al.*, 2006], or by protein and lipid synthesis in the roots themselves or in the associated mycorrhiza, since the conversion of carbohydrates to more reduced compounds result in ARQ >1.0 [*De Vries et al.*, 1974; *Hawkins et al.*, 1999; *Shane et al.*, 2004]. Another process suggested to occur in roots is dissolution of a fraction of the root-respired CO_2 in the xylem water and its transport to above ground tissues in the transpiration stream [*Aubrey and Teskey*, 2009; *Grossiord et al.*, 2012]. This CO_2 removal is expected to reduce ARQ_{root} during the daytime when transpiration is active. The ARQ associated with respiration in the rhizosphere also depends on the composition of the root exudates, which vary greatly in C_{ox} [*Bais et al.*, 2006]; ARQ will be above 1.0 when exudates are dominated by organic acids and below 1.0 when dominated by amino acids (Table 1, Fig. 1).

Respiration in tree stems is also based on carbohydrates with expected ARQ (ARQ_{ts}) of 1.0. However, the mean ARQ_{ts} measured from stem chambers in tropical, temperate, and Mediterranean trees was 0.59 and values of 1 were rarely observed [*Angert et al.*, 2012; *Hilman et al.*, 2019] (Fig. 1). Dissolution and transport of respired CO_2 via the xylem water stream is thought to influence the CO_2 efflux measured from tree stems [*Teskey et al.*, 2008]. However, CO_2 transport was found to have only a minor role in explaining low ARQ_{ts} in the investigated trees [*Hilman et al.*, 2019]. One indication for little effect of CO_2 transport in the xylem water was that ARQ_{ts} values measured in stem chambers were similar to those measured during incubation of stem tissues that were clearly detached from the xylem system and unaffected by transport. An alternative hypothesis for lower than expected ARQ_{ts} values is non-phototrophic CO_2 fixation by the enzyme phosphoenolpyruvate carboxylase (PEPC) [*Hilman et al.*, 2019], which was found to be highly abundant in young tree stems [*Berveiller and Damesin*, 2007; *Berveiller et al.*, 2007]. Photosynthetic fixation of CO_2 under tree bark would produce O_2 and

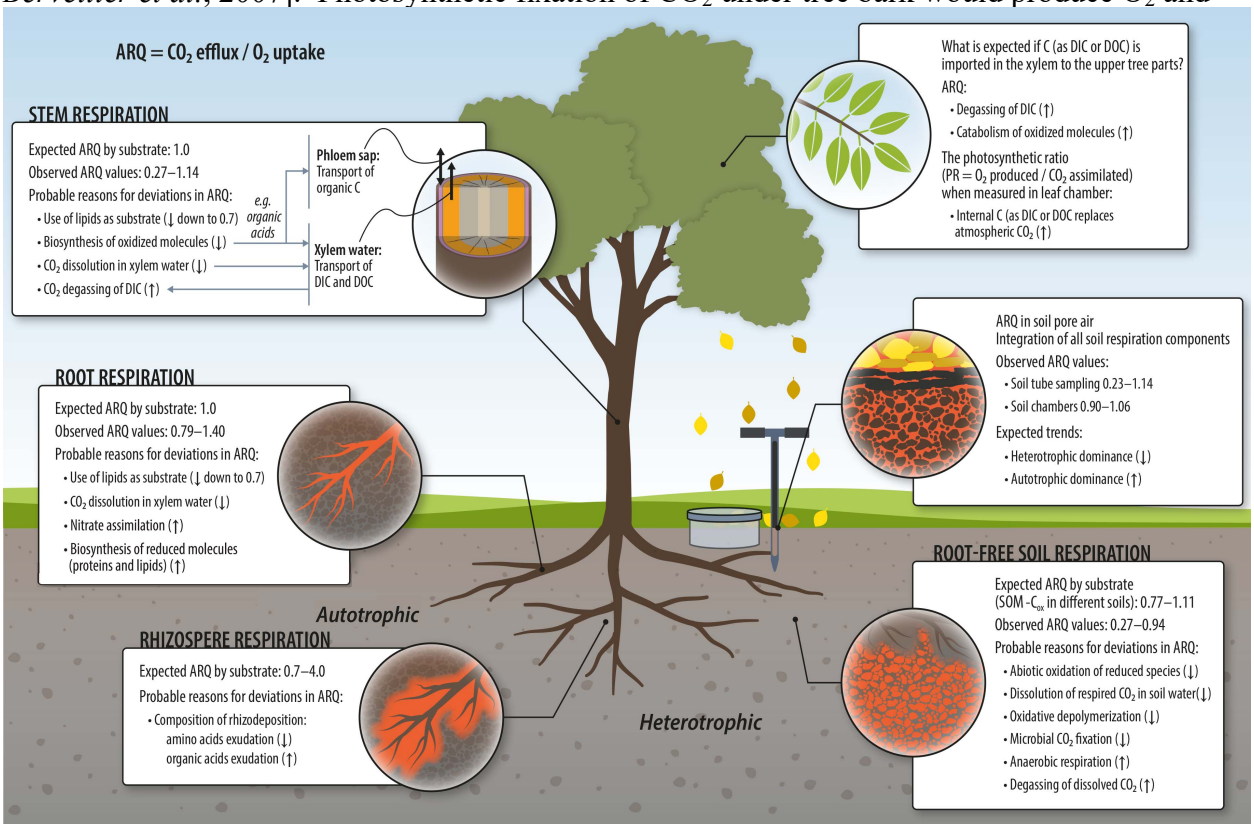


Figure 1 Observed ARQ in different ecosystem components with plausible explanations for deviations from the expected value. The expected ARQ is according to the putative-substrate stoichiometry (see Table 1): Carbohydrates for respiration in roots and stems; rhizodeposition composition for rhizosphere respiration, with values indicative of the end-members amino and organic acids; and soil organic matter composition for root-free soil respiration according to *Worrall et al.* [2013]. The observed ARQ values are taken from the literature: Stem respiration [*Angert et al.*, 2012; *Angert and Sherer*, 2011; *Hilman et al.*, 2019]; Root respiration [*Hawkins et al.*, 1999; *Rachmilevitch et al.*, 2006; *Shane et al.*, 2004]; Root-free soil respiration [*Angert et al.*, 2015; *Aon et al.*, 2001a; b; *Dilly*, 2001; 2003; *Dilly and Zyakun*, 2008; *Severinghaus*, 1995]; Soil pore air, tube sampling [*Angert et al.*, 2012; *Angert et al.*, 2015; *Hicks Pries et al.*, 2019; *Sanchez-Canete et al.*, 2018], soil chambers [Ishidoya et al., 2013; Seibt et al., 2004]. Included

also are the expected effects on ARQ and the photosynthetic ratio (O_2 produced/ CO_2 assimilated) if C is imported internally to the canopy.

1.2 ARQ as a tool to separate respiration sources in soil and ecosystem

Total soil respiration integrates heterotrophic respiration in root-free soil and the root-associated rhizosphere with autotrophic respiration by roots. Measurements of ARQ of total soil respiration (ARQ_{sa}) have been made in two ways. Most frequently, they have been estimated from the difference in the ratio of CO_2/O_2 in soil air pore space (sampled by a tube) compared to the CO_2/O_2 of overlying air, corrected for diffusivity differences [*Angert et al.*, 2015]. Using this method a wide range of values, between 0.23 and 1.14, were measured in a variety of forest soils from different biomes [*Angert et al.*, 2012; *Angert et al.*, 2015; *Hicks Pries et al.*, 2019; *Sanchez-Canete et al.*, 2018]. The second measurement monitored simultaneous changes in CO_2 and O_2 in the headspace of a chamber covering the soil surface. These measurements tend to have higher ARQ_{sa} values, ranging between 0.90-1.06 [*Ishidoya et al.*, 2013; *Seibt et al.*, 2004]. The large observed ARQ_{sa} variability can be attributed to two factors: variability in the relative weight of each of the soil respiration sources, and variability within each of the sources. For example, *Hicks Pries et al.* [2019] found strong seasonality in ARQ_{sa} in western US forest conifer stand with summer vs. winter values of 0.89 and 0.70, respectively. The seasonal variation, observed also in soil air $\delta^{13}CO_2$, was attributed to changes in respiratory substrates that switched between root-based respiration of more oxidized compounds during summer and bulk-soil respiration of more reduced compounds during winter (Table 1, Fig. 1). Similarly, greater contributions of autotrophic respiration and litter decomposition in shallower horizons that are not well-captured by soil gas sampling tubes might also explain the high ARQ_{sa} measured from soil chambers. Overall, different ARQ values for different components of soil (or ecosystem) respiration show promise for its use to partition respiration sources or identify transport of CO_2 between components.

While measured values of ARQ from soils and stems are often < 1, total ecosystem CO_2 and O_2 fluxes are nearly balanced (ARQ ~ 1). Direct estimates of the oxidative ratio (OR = 1/ARQ) using gas measurements in and above canopies ranged between 0.94 and 1.10 over diel and annual cycles [*Battle et al.*, 2019; *Ishidoya et al.*, 2013; *Seibt et al.*, 2004; *Stephens et al.*, 2007]. OR over longer time scales can be estimated by the ecosystem's C_{ox} [*Keeling*, 1988; *Masiello et al.*, 2008; *Severinghaus*, 1995]. According to a meta-analysis of total biomass and SOM-C_{ox}, OR is mostly between 1.00 – 1.13 in 16 global biomes [*Worrall et al.*, 2013]. Therefore, the low ARQ measured in soils and tree stems must be balanced, at least over longer time scales, by higher ARQ measured elsewhere.

The fact that ARQ_{ts} is almost always lower than the expected value of 1 from substrate carbohydrates may indicate a process that retains respired CO_2 within the stem [*Hilman et al.*, 2019]. If the CO_2 retention in the stem is due to PEPC re-fixation, biosynthesis of compounds more oxidized than carbohydrates like organic acids are expected [*Lambers et al.*, 2008]. The catabolism of organic acids results in ARQ >1 (Table 1) that will balance the observed low ARQ_{ts} where CO_2 is fixed. The high ARQ may appear in the rhizosphere, if organic acids are exported via the phloem to the roots and transferred to the soil as root exudates [*Hoffland et al.*, 1992; *Shane et al.*, 2004]. Alternatively, the organic acids might be transported upwards in the xylem water [*Schill et al.*, 1996] and contribute to respiration in the canopy. For example, the

organic acid malate can contribute C to leaf photosynthesis [*Hibberd and Quick*, 2002], and alter the photosynthetic ratio evaluated by leaf O_2/CO_2 fluxes.

Another process proposed for CO_2 retention in the stem is CO_2 dissolution in the xylem water. In this case, CO_2 degassing in higher parts of the tree will result in ARQ values that are locally >1.0. In addition, dissolved CO_2 may contribute to canopy photosynthesis [*Bloemen et al.*, 2013; *Stringer and Kimmerer*, 1993] in which case it will also affect the photosynthetic.

2 Materials and Methods

In this study, we focused on the seasonal variation of ARQ in soils and tree stems. Seasonal measurements at ~1.5 months intervals of in situ ARQ_{sa} estimated from soil gas tubes and a diffusion model, and ARQ_{bs} and ARQ_{ts} using soil and tree stem incubations were conducted in a Mediterranean oak system. After initially performing bulk-soil incubations (ARQ_{bs}) at room temperature, we realized that the different temperatures in the field may affect measured ARQ_{bs}. We therefore performed an additional experiment to test the temperature effect on ARQ_{bs} and used this to correct the ARQ_{bs} results. The evaluation of the control of physical variables (water availability and temperature) on ARQ seasonal variability was performed using a backward selection technique for multiple regressions. ARQ_{sa} was expected to be higher than ARQ_{bs} due to the contribution of root respiration with higher ARQ than in the bulk soil. A direct test was done by comparison of ARQ_{sa}, ARQ_{bs}, and ARQ_{root} sampled at the same time. Additional experiments were conducted to investigate the potential of Fe^{2+} and Mn^{2+} oxidation to reduce ARQ_{bs}. Following the hypothesis that low ARQ_{ts} is the result of CO_2 re-fixation and production of organic acids, we expected anti-correlation between ARQ_{ts} and ARQ_{sa} if the produced organic acids are secreted finally to the rhizosphere as exudates.

2.1 Study site

The study took place in Odem Forest, located 950 m a.s.l, 33°13’ N, 35°45’ E. The climate is Mesic Mediterranean with a mean annual precipitation of 950 mm and summer and winter mean temperatures of 21.3º C and 7.3ºC, respectively. The dominant tree species are the evergreen *Quercus calliprinos* Webb (about 75% of the woody cover area) and the winter-deciduous *Quercus boissieri* Reut. (15%) [Kaplan and Gutman, 1996]. *Q. calliprinos* is the dominant tree in the Mediterranean scrub in Israel, while *Q. boissieri* grows mainly in altitudes above 500 m a.s.l [Kaplan and Gutman, 1996]. The soil was formed on basaltic bedrock and is classified as Eutric Lithosol in the FAO classification system and as Lithic Xerorthent in the USDA classification system. The soil pH is 6.6 and the organic C content in the top 10 cm is 12% [Gross and Angert, 2017].

2.2 Experimental design

2.2.1 Seasonal measurements

Seasonal sampling took place in ten campaigns between February 2017 and May 2018. Soil air was sampled from 1/2" (OD) stainless steel tubes closed at the bottom end, and perforated near the bottom, that were hammered into the soil. The samples of soil air were collected from a depth of 15 ± 4 cm in pre-evacuated ~3.6 mL glass flasks with Louwer™ O-ring high-vacuum valves. Before sampling, the dead volume in the tubing and flask necks was purged with soil air by a

plastic syringe equipped with a two-way valve. A total of 120 samples were taken near each tree species (2 replicates x 2 samples x 3 trees x 10 campaigns). Every tree was sampled only once since sampling caused some disturbance to the soil and the stem (see below).

Surface soil from 0-10 cm depth was collected with a trowel and stored in a plastic bag. A total of 30 samples were taken near each tree species by pooling from two places near each tree (3 trees x 10 campaigns). Soil moisture was measured gravimetrically on ~3 g subsamples (available only for the last 6 campaigns). For bulk soil incubation experiments, the soil was sieved to 2 mm (except on January 2018 sampling when the soil was too wet and sticky to allow sieving), and a subsample of 3 g was incubated overnight in 6 mL glass test tubes connected to ~3.6 mL glass flasks by Ultra-Torr fittings (Swagelok, Solon, OH, USA). The gas in the headspace had initial mean atmospheric values (20.95% O_2, 0.04% CO_2). Incubations were conducted usually two days after soil collection at room temperature.

For estimating ARQ_{ts} we performed stem tissue incubations. This method was shown to give similar ARQ values as the stem-chamber method in three species including the oak *Quercus ilex* [Hilman et al., 2019]. We decided to incubate only the phloem and cambium tissues since they are the most metabolically active tissues in the stem [*Bowman et al.*, 2005], and since transport in the phloem is the pathway for C to flow from the stem to the roots. Cores were extracted using a 1.0 cm diameter cork borer, at 20 cm and 130 cm above the soil surface. A total of 60 samples were taken from each tree species (2 stem positions x 3 trees x 10 campaigns). We removed from the cores the outer bark and sapwood sieves, and further cut the cores to fit into the 3.6 mL glass flask neck. For the incubations, we plugged the neck with a rubber stopper to create a gas-tight headspace with initial mean atmospheric values. The incubations started immediately after harvesting and lasted 3-4 hours in the dark and at environmental temperatures. Metabolism in stem cores changes rapidly after harvesting; in a previous study ARQ_{ts} increased with time after harvesting from 0.4 to values closer to 1.0 while the O_2 uptake rate was maintained, indicating an increase in CO_2 efflux over time [Hilman et al., 2019]. The increased ARQ_{ts} may provide evidence for gradual inhibition of PEPC activity that fixed respired CO_2 early during the incubation, but became less efficient as its own products accumulated. To observe potential temporal changes in ARQ_{ts}, the tissues were re-incubated 24 hours after harvesting (ARQ_{ts24}) for the same duration at room temperature. In the 24 h storage time stem tissues were wrapped with moist gauze cloth to avoid desiccation.

2.2.2 Comparison of roots, bulk soil, and soil air ARQ and temperature effect

To evaluate the potential for ARQ to partition the contributions of autotrophic and heterotrophic sources to total soil respiration, and to test the sensitivity of ARQ_{bs} to incubation temperature, we sampled additional trees. During January 2019 we measured ARQ_{sa}, ARQ_{bs}, and ARQ_{root} near three additional trees from each species. For ARQ_{root} fine roots (< 2 mm), which showed the highest specific respiration rates among the root diameters tested, were excavated [*Chen et al.*, 2010; *Desrochers et al.*, 2002; *Pregitzer et al.*, 1998]. To test possible effects of root-surface microbial communities on ARQ_{root}, soil was washed thoroughly from one subsample of roots before incubation (n = 3), while a second subsample (n = 3) was incubated with the surrounding soil. Roots were incubated shortly after harvesting in the dark in a set-up of two 3.6 mL glass flasks connected by Ultra-Torr fitting, and kept at ~7^{o}C to represent field condition (field soil temperature was 6-8^{o}C). Since we expected low respiration rates incubations lasted 24 h. We also included in the comparison coarse roots of *Q. calliprinos* (< 1 cm in diameter) collected in

March 2018. Bulk soil incubations were conducted at temperatures of 6, 22, and 30 ^{o}C and lasted 68-90 h (2 samples x 3 trees). The Q_{10}, the factor by which respiratory flux rises with a 10 ^{o}C increase, was calculated using the function Q_{10} from the R package *respirometry* [R Core Team, 2019]. We also present ARQ_{bs} values for soils sampled in March and May 2018, when soil temperatures were 1°C and 22^{o}C, respectively (n = 1).

2.2.3 Evaluation of the effect of abiotic O_2 uptake on bulk soil ARQ

Two additional soil incubation experiments were undertaken to investigate the potential for abiotic O_2 uptake to affect ARQ_{bs}. In the first experiment we tested the response to temporary anaerobic conditions with un-screened soils (for maintaining their structure). Mason jars (1 L) with a small volume of soil (~150 ml, n = 3) and jars with large soil volume (~550 ml, n = 3) were incubated for 13 days, to create low O_2 concentrations. Headspace [O_2] was measured by the end of the incubation, and soils were sampled for [Fe^{2+}] and [Mn^{2+}]. The soils were then ventilated for 1.5 hours, before an overnight incubation. Air and soil samples were measured again at the end of this incubation. The soil moisture measured at the beginning of the experiment was 31% by weight. The soil [Fe^{2+}] was measured by the Ferrozine method [*Liptzin and Silver*, 2009]. The soil samples were sieved to 2 mm, and extracted by 0.5 M HCl immediately at the end of the incubation experiments. The soil [Mn^{2+}] was measured by assuming that HCl-extractable Mn, which was quantified by ICP (7500cx Agilent technologies, Santa Clara, CA, USA), predominantly represents Mn^{2+} [*Keiluweit et al.*, 2018].

In a second experiment we tracked ARQ_{bs} during a wetting-drying cycle, and measured [Fe^{2+}] and soil moisture during the soil drying. Ultra-Torr Tee fittings (Swagelok, Solon, OH, USA) were used for the incubation, connecting a test-tube with soil, a test-tube with a drying-agent (magnesium perchlorate), and a 3.6 ml flask equipped with Louwer™ O-ring high-vacuum valve. We incubated 2-mm sieved soil (n = 1) and un-sieved soil (n = 1). After every incubation the flask was closed and removed, the system was ventilated for 1 hour and then new flask was attached. The first incubation was used to determine the basal ARQ_{bs} and respiration rate (O_2 uptake). The soil was then dried for 17 days, wetted, and dried again for 26 days. Soil wetting was roughly equivalent to a rainfall event of 20 mm. The destructive Fe^{2+} and soil moisture measurements during the soil drying were done for the sieved soil, after re-wetting it to the same degree. We report the relative respiration rate (RR) as the ratio between the O_2 uptake in each incubation to the basal rate.

2.4 Gas analysis

The [O_2] and [CO_2] of the air samples were measured in the laboratory by a closed system (The "*Hampadah*" [*Hilman et al.*, 2019]). The system is based on two analyzers: an infra-red gas analyzer (IRGA) for CO_2 measurement (LI 840A LI-COR; Lincoln, NE, USA) and a fuel-cell based analyzer (FC-10; Sable Systems International, Las Vegas, NV, USA) for measuring O_2, and is fully automated.

For measuring [CO_2] and [O_2] from the Mason jars we equipped each lid with a septum. Air from the headspace was sampled by plastic syringe with needle and injected into a flow-through CO_2 (K33 ICB 30% CO_2 Sensor, CO_2 Meter, Inc) and O_2 (Fibox 3, PreSens-Precision Sensing) sensors, connected by plastic tubing. The O_2 sensor is a quenching based optical fiber (optode) that reads the fluorescence from a sensing "spot". We placed the "spot" in a 3 mm clear plastic aperture in an opaque lid of a custom-made 2-cm diameter flow-through cell, which made

from 4 mm thick aluminum base (to stabilized the temperature). From the outside of the aperture a connector for the optical fiber that reads the "spot" fluorescence was fixed. The same air was injected to pre-evacuated ~3.6 mL glass flasks for comparison with the "*Hampadah*" method.

2.3 ARQ and respiratory fluxes estimations

ARQ_{sa}, the CO_2 efflux/O_2 uptake in soil respiration, was calculated from the measured gases concertation using the following equation [*Angert et al.*, 2015]:

$$\mathrm{ARQ} = \frac{D_{CO2}\times([CO_2]_s-[CO_2]_a)}{-D_{O2}\times([O_2]_s-[O_2]_a)} = -0.76\times\frac{\Delta CO_2}{\Delta O_2} \quad (1)$$

Where D_{co2}, $[CO_2]_s$, and $[CO_2]_a$ are respectively the effective diffusivity of gaseous CO_2, and the CO_2 concentrations in the soil (measured value) and in the ambient air (assumed to be 0.04%). ΔCO_2 is therefore the difference in $[CO_2]$ between the soil and the atmosphere. The same definitions hold for O_2, where the concentration in the ambient air assumed to be 20.95%. Since the O_2 flux is opposite in direction to the CO_2 flux we added negative sign for convenience. The effective diffusivity D depends on the structure of the pore spaces and on the diffusivity in air. It can be assumed that the structure is identical for CO_2 and O_2, therefore the ratio D_{co2}/ D_{o2} depends only on the CO_2/O_2 diffusivity ratio in air, which is 0.76 [*Massman*, 1998].

Eq. 1 is somewhat analogous to the Davidson [*Davidson*, 1995] equation that estimates the respired $\delta^{13}CO_2$ in soil from soil-air discrete sample. The equation corrects for the faster diffusion of $^{12}CO_2$ (in our case O_2) that enriches the soil air with the slower-diffusing $^{13}CO_2$ (in our case CO_2), and for difference in $\delta^{13}C$ and $[CO_2]$ between the soil and the air above the soil. The Davidson equation accounts for diffusion and diffusive mixing and thus yields the same respired $\delta^{13}CO_2$ regardless soil depth [*Bowling et al.*, 2015; *Egan et al.*, 2019]. Eq. 1 is expected to similarly account for gas-mixing effects with depth [*Angert et al.*, 2015]. Indeed, ARQ_{sa} was similar when measured at depths of 10, 30, and 60 cm (0.3, [*Sanchez-Canete et al.*, 2018]) and at 30 and 90 cm (0.8, [*Hicks Pries et al.*, 2019]). Moreover, as ΔCO_2 and ΔO_2 are calculated with respect to atmospheric air, advective mixing of soil air with the above air is not expected to change the measured ARQ_{sa}. Nonetheless, since such mixing will violate the diffusional steady-state between different soil depths we avoided sampling in days with high wind speeds (> 4 m s^{-1}).

ARQ_{bs}, ARQ_{ts}, and ARQ_{ts24} were calculated by the ratio between $[CO_2]$ and $[O_2]$ net percent changes in the incubation headspace. ARQ_{bs} values were further corrected for CO_2 dissolution since the large volume of water in the bulk soil samples collected during winter and the fairly high pH value for non-calcareous soil (6.6) are expected to cause to some of the respired CO_2 to convert into DIC. DIC (mmol/L), the sum of dissolved CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}, was calculated using Eq. 2 [*Stumm and Morgan*, 1996]:

$$\mathrm{DIC} = P_{CO2}\times k_h\times\left(1+\frac{k_1}{10^{-pH}}+\frac{k_1\times k_2}{(10^{-pH})^2}\right) \quad (2)$$

where P_{CO2} is the partial pressure of gaseous CO_2 (= $[CO_2]/100$), k_h is Henry's constant that states the amount of dissolved CO_2 and H_2CO_3 in the water, and k_1 and k_2 are the first and second acidity constants, respectively. The constants are temperature dependent and calculated according to the incubation temperature [*Harned and Davis*, 1943; *Harned and Scholes Jr*, 1941]. To estimate the addition of DIC during incubation we assumed the initial DIC was in

equilibrium with atmospheric [CO_2] of 0.04%, and the final DIC in equilibrium with the final [CO_2]. The net change in the calculated DIC (ΔDIC) was converted to gaseous CO_2 equivalent and added to the measured $[CO_2]_{measured}$, yielding the corrected $[CO_2]_c$:

$$[CO_2]_c = [CO_2]_{measured} + 100 \times \frac{\Delta DIC \times W \times 22.4}{V_{HS}} \quad (3)$$

where W is the absolute amount of water in the sample (L) and V_{HS} is the headspace volume (mL). When soil moisture data were unavailable, we estimated its value from the relation between the available soil moisture data and rainfall in the last 3 weeks. The term 22.4 converts the units of the dissolved CO_2 (mmol) to mL gas.

The O_2 uptake and CO_2 efflux from the bulk-soil incubations were calculated using the equation (nanomole gas $g.DW^{-1}$ min^{-1}):

$$Flux = \frac{\Delta Gas \times V_{HS} \times BP}{t \times M \times I_t \times 8.314 \times 10^{-3}} \quad (4)$$

where ΔGas is net percent change in the gas concentration ([O_2] or [CO_2]) during the incubation, BP is the local barometric pressure (hPa), t is the temperature (k), M is the soil dry weight (g), I_t is the incubation time (min), and 8.314×10^{-3} is the ideal gas constant (mL hPa k^{-1} $nanomol^{-1}$). Soil samples were oven-dried (105°C, 24 h) for dry weights.

2.5 Statistical analysis

All comparisons were conducted using one-way analysis of variance (ANOVA) and a *t*-test, after assuring homogeneity of variances using Bartlett's test. For unequal variances we used a Welch's test and nonparametric comparisons with Wilcoxon method. Significant differences were determined at $P < 0.05$. The relations of the seasonal ARQ and bulk-soil O_2 uptake values with physical parameters were tested using backward selection technique for multiple regressions, including estimates of the interactions between each two factors. The tested parameters were: soil moisture (available for the last 6 out of 10 campaigns), the number of days passed since the last rain event, the accumulated rain in the 3 weeks prior to sampling, and soil temperature at 10 cm depth. Rainfall and temperature were measured in the nearby metrological station of El rom (data courtesy of Meteo-Tech Ltd. Meteorological Services). We used linear regressions not only to evaluate the relationship of dependent and independent variables, but also to describe correlation between ARQ_{ts} and ARQ_{sa}. All statistical analysis was done using JMP (JMP®, JMP Pro 13, SAS Institute Inc., Cary, NC, USA).

3 Results

3.1 Seasonal measurements

The seasonal ARQ measurements are presented in Figure 2. The overall mean ± SE values (range of means per species per date) of ARQ_{sa}, ARQ_{bs} (without correction for CO_2 dissolution in water), ARQ_{ts}, and ARQ_{ts24} were respectively 0.76 ± 0.02 (0.60-0.92), 0.65 ± 0.02 (0.47-0.80), 0.39 ± 0.03 (0.19-0.70), and 0.68 ± 0.04 (0.42-1.08). The dissolution correction for ARQ_{bs} values increased the mean value and range to 0.72 (0.51-0.88). The correction had some sensitivity to the parameters pH, W (soil water content), and V_{HS} (incubation headspace) (Eq. 2-3). The decrease of V_{HS} in 10%, and the increase of W in 10% and the pH from 6.6 to 6.8 resulted in an increase of the overall mean to 0.76. Varying the parameters in the same magnitude but in

446 opposite direction yielded overall mean of 0.69. The weighted mean of the corrected ARQ_{bs}
447 (using O_2 uptake rates for weighting) further increased the mean value to 0.75 (0.53-0.90). From
448 this point on in the paper ARQ_{bs} values will refer to values corrected for CO_2 dissolution.
449 Significant difference between species were observed only in ARQ_{ts} ($P = 0.0015$, *Welch* test,
450 both stem heights pooled together) with mean values of 0.46 ± 0.03 and 0.32 ± 0.02 for *Q.*
451 *boissieri* and *Q. calliprinos*, respectively. Marginal significance ($P = 0.06$, *t* test) was observed
452 for the weighted ARQ_{bs} where *Q. calliprinos* was higher than *Q. boissieri* (0.77 vs. 0.72).

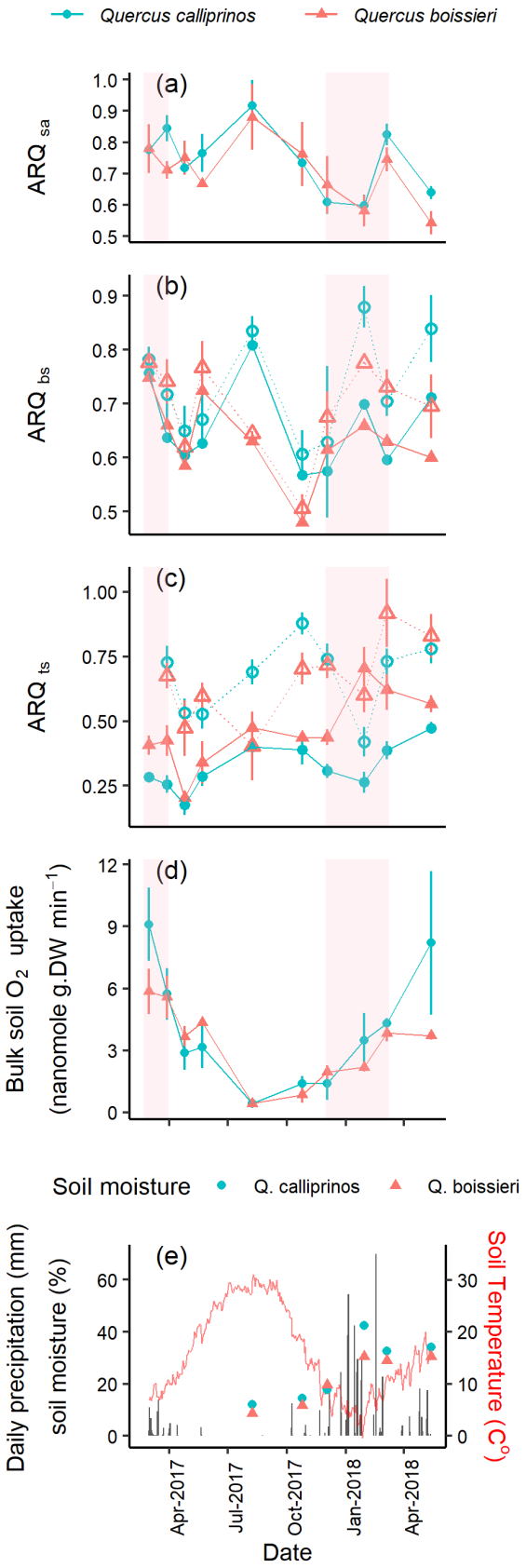


Figure 2. Seasonal measurements of (a) ARQ_{sa} ± SE, the ratio of CO_2 efflux/O_2 uptake measured for soil air in depth of 15 ± 4 cm (n = 3); (b) ARQ measured from bulk soil incubation (ARQ_{bs}) where full markers with soil line indicate the measured values and empty markers with dashed lines indicate the dissolution corrected values (n = 3); (c) the mean ARQ measured for incubated stem tissues (ARQ_{ts}) extracted from at 20 and 130 cm above the ground (ARQ_{ts}, full markers indicate incubations conducted directly after the core was harvested; empty markers with dashed lines indicate incubations conducted 24 h after harvest) sampled 20 and 130 cm above ground, (d) the O_2 uptake rate of the incubated bulk soils, (e) daily precipitation (black bars) and soil temperature (blue line) measured by adjacent meteorological station and the soil moisture in the site. Shaded periods indicate winter dormancy of the deciduous *Q. boissieri*. Soil sampling was conducted underneath the trees. Error bars represents standard errors.

3.2 Bulk soil measurements

The seasonal variability of ARQ_{bs} was not explained by the tested physical parameters in the backward selection technique. When temperature was tested individually it had positive effect on ARQ_{bs} between 1-6 and 22^{o}C, while between 22 and 30^{o}C ARQ_{bs} values were rather stable (Fig. 3a). At the highest temperatures we observed species effect with higher values in the *Q. calliprinos* (Fig.3a). The *Q. calliprinos* also had higher CO_2 and O_2 fluxes, greater sensitivity to temperature (higher Q_{10} values, Fig. 3b,c), and greater soil moisture than the *Q. boissieri* (Fig. 3d). For correction of the seasonal incubations conducted at room temperature to field soil temperature, we used a logarithmic fit ($R^2 = 0.78$) equation for the relation between $T_{incubation}$ (the temperature in which the incubation took place (C^{o})) and ARQ_{bs} for both species:

$$ARQ_{bs} = 0.13 \times \ln(T_{incubation}) + 0.36 \quad (5)$$

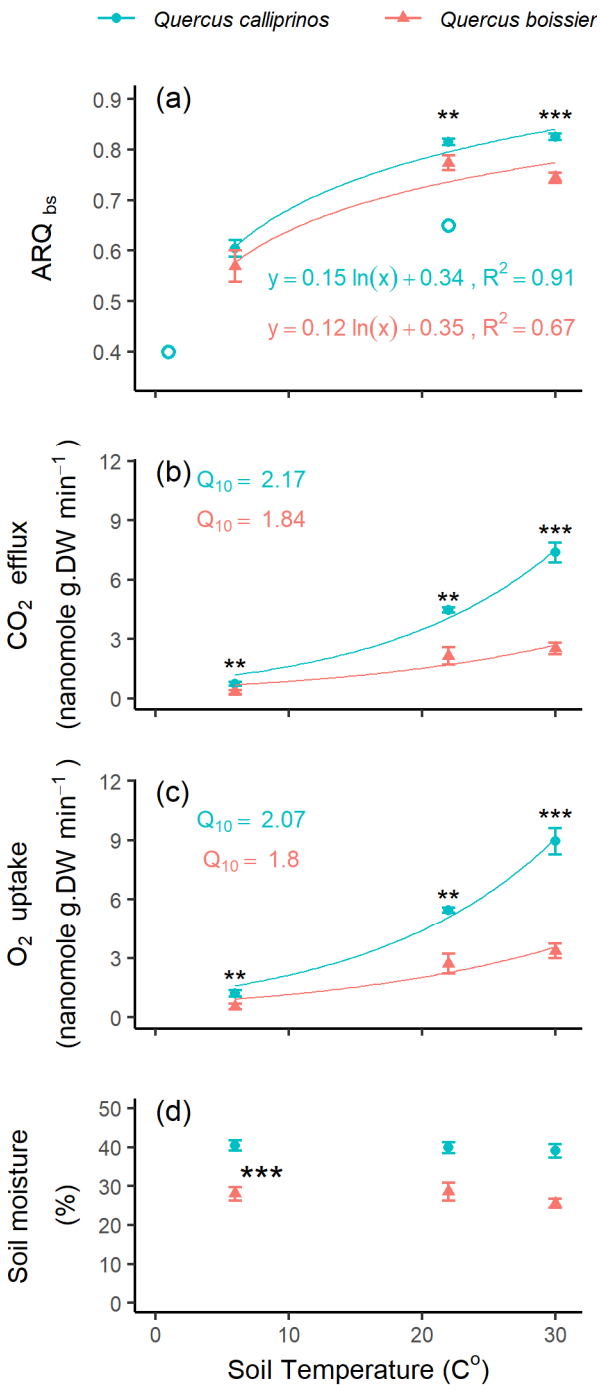


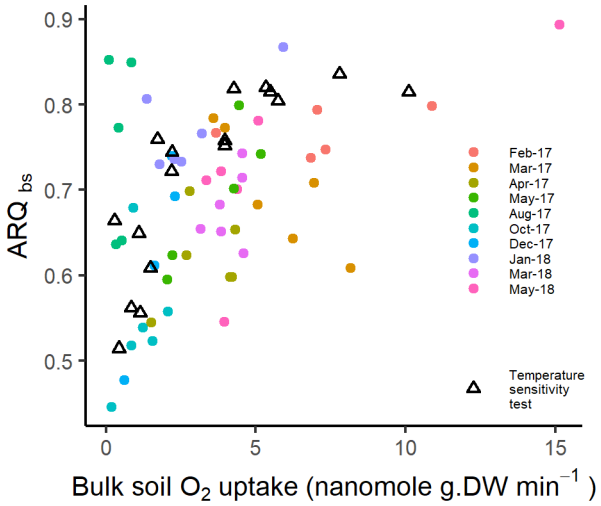
Figure 3. Results (mean ± SE) from bulk soil incubations at different temperatures. Filled symbols represent soils collected on January 2019 underneath three trees from each species with two soil samples per tree (n=6). Empty symbols represent soils collected on March and May 2018. (a) ARQ_{bs} (ratio of CO_2 efflux/O_2 uptake) with logarithmic fit, (b) the CO_2 efflux rates (after CO_2 dissolution correction) and the calculated temperature coefficient Q_{10}, (c) the O_2 uptake rates and the calculated Q_{10} values, and (d) the gravimetric moisture of the soils. Asterisks indicate significance difference between species (** - $P < 0.01$, *** - $P < 0.001$) in *t*

485 test, expect for the CO_2 and O_2 fluxes at 22°C where Welch test was used. For soil moisture
486 comparison was made for all temperatures together.

487 A trend of higher ARQ_{bs} values with higher bulk-soil O_2 uptake rates was observed for
488 both the seasonal measurements and the temperature-controlled incubations (Fig. 4). In addition,
489 variability declined with increasing O_2 uptake rates. The bulk-soil O_2 uptake rate showed a
490 strong seasonal cycle, with maximal uptake rates during spring (March-May) and minimal rates
491 during the end of the summer (August-October) (Fig. 2d). The uptake rates of the two species did
492 not differ significantly ($P = 0.766$, *t* test). A reciprocal effect on bulk-soil O_2 uptake rate
493 (nanomole O_2 g.DW min^{-1}) was found between soil moisture (M) and soil temperature (T_{soil}).
494 The effect is described by the following equation:

495 $$O_2 \text{ uptake} = 17.50\,M + 0.09\,T_{soil} + (T - 14.5) \times (M - 0.24) \times 0.59 - 2.79 \quad (6)$$

496 The actual respiration rate versus the equation predicted respiration gives $R^2 = 0.94$ ($P <$
497 0.0001). A significant linear relation was found also between bulk-soil O_2 uptake and the number
498 of days elapsed since the last rain event ($R^2 = 0.4$, $P = 0.005$). Adding this effect to the prediction
499 formula does not improve R^2 which remains 0.94 (no reciprocal effect was found in relation to
500 this parameter). A correlation coefficient R^2 of 0.75 ($P < 0.001$) was calculated while assuming
501 M is the only driving factor on bulk-soil O_2 uptake.
502



503

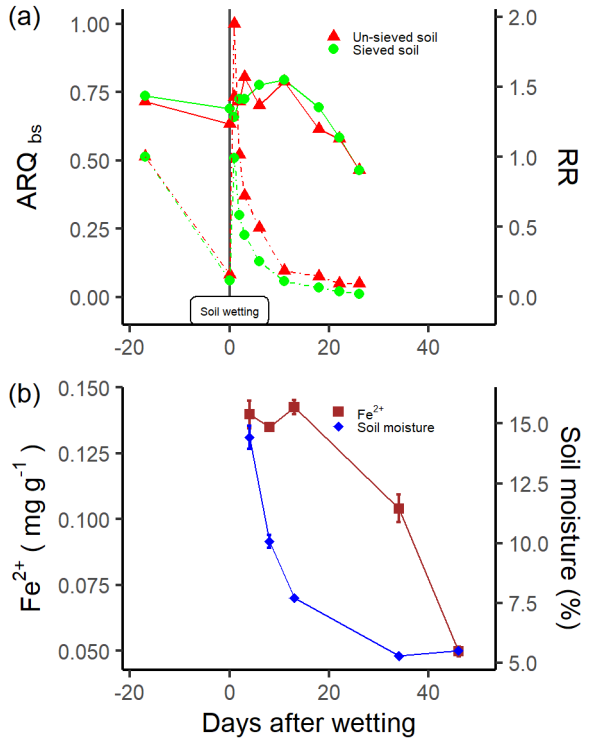
504 Figure 4. Scatter plot of ARQ (the ratio of CO_2 efflux/O_2 uptake) measured from bulk soil
505 incubations (ARQ_{bs}) and the O_2 uptake rate of the incubated bulk soils. Results are grouped by
506 the month of sampling for the seasonal measurements (colored circles) and temperature

507 **3.2.1 Relation between ARQ_{bs} and Fe^{2+}**

508 After the first 13-days of incubation the average [O_2] ± SD of the incubation jars with the large
509 and small soil volumes were 0.90 ± 0.44% and 7.25 ± 0.07%, respectively (n = 3). In agreement,
510 [Fe^{2+}] in the jars with large soil volume was higher than measured for the small soil volume jars
511 (0.89 ± 0.24 vs. 0.05 ± 0.01 mg g^{-1} soil, respectively). In the subsequent incubation, performed
512 after ventilation of 1.5 hours aimed to increase the [O_2] in the jars, a sharp decrease in [O_2] was
513 observed in the large soil volume jars from an ambient value of 20.95% to value of 4.77 ±

514 0.21%, while ARQ was 0.37 ± 0.01. The [Fe^{2+}] dropped from 0.89 ± 0.24 to 0.21±0.04 mg g^{-1}
515 soil. The [Mn^{2+}] was 1.27 mg g^{-1} soil, and did not significantly change during this incubation. In
516 the small soil volume jars the [O_2] decreased from 20.95% to 19.80 ± 0.26%, ARQ was
517 0.74 ± 0.02, and [Fe^{2+}] did not change from the initial value of 0.05 mg g^{-1} soil. Taking into
518 account the different soil volumes, the rate of O_2 uptake was 2.9-fold faster in the large soil
519 volume jars than in the small soil volume jars. The [CO_2] and [O_2] determined by the sensors in
520 this experiment were highly consistent with the *Hampadah* measurement (R^2 of 0.997 and 0.975
521 in linear regression with slopes of 1.01 and 1.01, respectively).

522 The soil wetting-drying experiment induced variations in ARQ_{bs}, RR, and [Fe^{2+}] (Fig. 5).
523 RR peaked in the day of soil wetting and then gradually decreased. Following the soil wetting
524 ARQ_{bs} increased during 11 days from 0.63-0.69 to 0.79-0.80 and then decreased during 15 days
525 to 0.46. [Fe^{2+}] values of ~0.14 mg g^{-1} were measured during the first 13 days after soil wetting, at
526 soil moisture values of 14.4%-7.7%. After the 13th day [Fe^{2+}] decreased to 0.10 mg g^{-1} in the 34th
527 day and to 0.05 mg g^{-1} in the 46th day.



528
529 Figure 5. Results from soil drying-rewetting experiment. The day of the rewetting is day 0. (a)
530 ARQ_{bs} (ratio of CO_2 efflux/O_2 uptake) in solid lines and relative respiration rate (RR) in dashed
531 lines for un-sieved and sieved (2 mm) soils. Each data point represents one measurement without
532 replicates. (b) The concentration of Fe^{2+} (mg g^{-1}) and the gravimetric moisture of the sieved soil.
533 Following the experiment presented in panel a, the same sieved soil was wetted to the same
534 moisture. Each data point represents mean of duplicate sub-samples taken from the drying soil.
535 Error bars are the standard deviations.

536 **3.3 Soil air measurements**

537 Concentrations of CO_2 and O_2 in the soils in single tube samplings ranged from 0.17 - 2.25% and
538 20.79 - 18.14%, respectively. The lowest O_2 concentrations were measured during January 2018

after 163 mm of precipitation over the previous 3 weeks. The seasonal variability of ARQ_{sa} was explained by the water related parameters M, the number of days passed since the last rain event (D), and accumulated rain in the 3 weeks prior to sampling (R) in the backward selection technique. A reciprocal effect was found between the last two factors. The statistical model is defined by the equation:

$$ARQ_{sa} = 0.47M + 0.02D + 0.004R + (D - 18) \times (R - 58.5) \times 3 \times 10^{-4} + 0.24 \quad (7)$$

With $P = 0.0002$ on F test and the correlation between the actual and predicted soil ARQ gives R^2 of 0.94. The effect of T_{soil} is small testing all-year time scale and its addition to the prediction formula has a minor contribution to the coefficient of determination. However, when omitting from the analysis data collected during late winter and spring and including only data from May 2017 – Jan 2018, ARQ_{sa} is found to be strongly dependent in temperature ($R^2 = 0.92$, $P < 0.0001$). The relation is given by the linear equation: $ARQ_{sa} = 0.01 \times T_{soil} + 0.6$.

3.4 Comparison of ARQ in bulk soil, roots, and soil air

The mean ARQ_{root} per species per date ranged between 0.73-0.96 (Fig 6a). The root washing treatment in roots collected in January 2019 did not affect ARQ_{root} ($P = 0.99$, *t* test) therefore results were pooled together. ARQ_{sa} values were always lower than ARQ_{root} and higher than ARQ_{bs} (Fig. 6a). Assuming root and bulk-soil respiration are the only end members affecting the soil pore space air, their relative contributions could be estimated using a simple mixing model, where $ARQ_{sa} = X \times ARQ_{root} + (1 - X) \times ARQ_{bs}$ (Fig. 6a).

The seasonal ARQ_{bs} measurements were conducted in room temperature and not in the actual field soil temperature. Thus, for comparison of ARQ_{bs} and ARQ_{sa} (measured at field conditions) ARQ_{bs} has to be corrected to field temperature. We first averaged values of both species at each measurement date. Then the intercept term b from Eq. 5 was modified:

$$b = 0.36 - (ARQ_{t=room} - ARQ_{bs\_measured}) \quad (8)$$

Where 0.36 is the calculated intercept as appears in Eq. 5, $ARQ_{t=room}$ is the expected ARQ according to Eq. 5 and the room temperature (varied slightly between measurements), and $ARQ_{bs\_measured}$ is the measured ARQ in the bulk-soil incubation, corrected to CO_2 dissolution. The bulk soil ARQ values reported in Figure 7b were calculated with the equation:

$$ARQ = 0.13 \times ln(T_{soil}) + b \quad (9)$$

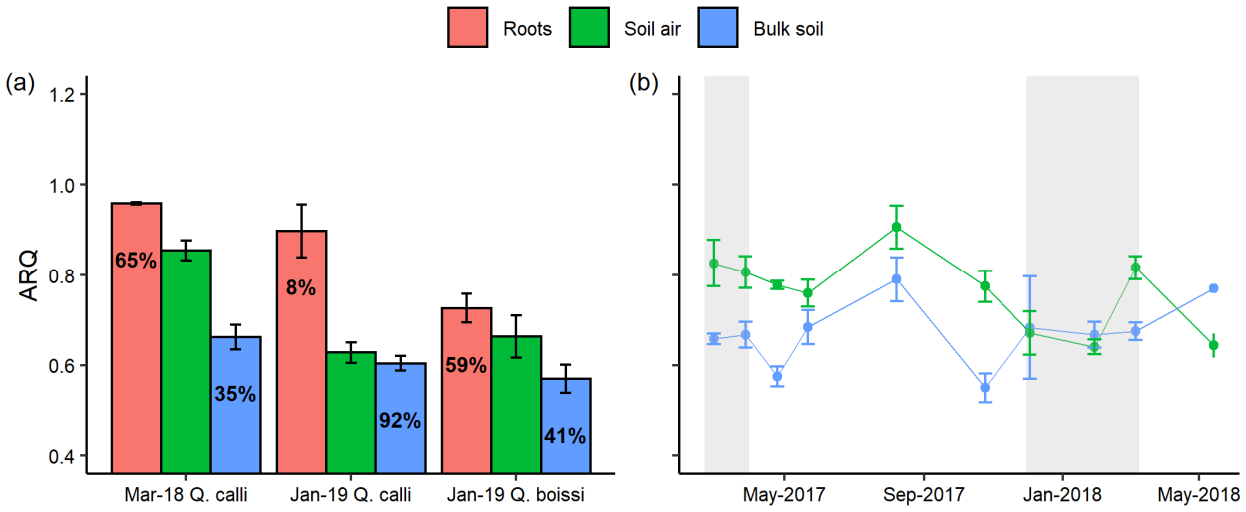


Figure 6. (a) A comparison of ARQ (ratio of CO_2 efflux/O_2 uptake) values (mean ± SE) measured from root incubations (n = 2, 6, 6), soil air (n = 3), and bulk-soil incubations (n = 3). The x axis indicates the date of sampling and the tree species. The relative contributions (%) of roots and bulk-soil respiration to the total soil respiration are indicated in the bars. The contributions were calculated using the equation $ARQ_{sa} = X \times ARQ_{root} + (1 - X) \times ARQ_{bs}$. (b) The seasonal course of ARQ means (± SE, n = 6) of both tree species, where the bulk soil values are temperature corrected according to Eq. 9. Shaded periods indicate winter dormancy of the deciduous *Q. boissieri*.

3.5 Tree stem measurements

The strongest effect on ARQ measured in tree stems was time; ARQ_{ts} values measured in incubations started immediately after tissues extraction were much lower than ARQ_{ts24} measured in incubation started 24 h after the extraction (Fig. 2c). The overall ARQ_{ts} and ARQ_{ts24} means (±SE) for the *Q. boissieri* were 0.46 ± 0.03 and 0.68 ± 0.03, respectively ($P < 0.0001$, *Welch* test), and for the *Q. calliprinos* 0.32 ± 0.02 and 0.67 ± 0.03, respectively ($P < 0.0001$, *Welch* test). The sampling height of the stem cores (20 and 130 cm) had no significant effect when analyzed separately for ARQ_{ts} and ARQ_{ts24} in both species ($P = 0.14$ at the minimum).

In general, the deciduous *Q. boissieri* exhibited greater seasonal variability than the evergreen *Q. calliprinos*. The *Q. boissieri* ARQ_{ts} values during winter exfoliation were significantly higher than the *Q. calliprinos* (0.51 ± 0.02 vs. 0.30 ± 0.02, $P < 0.0001$, *Welch* test), while during the foliated period higher *Q. boissieri* values were not significant (0.41 ± 0.03 vs. 0.34 ± 0.03, $P = 0.11$, *t* test). In contrast, the species did not differ in their ARQ_{ts24} values ($P = 0.66$, *t* test) also during winter exfoliation ($P = 0.07$, *Welch* test).

The seasonal variability of the *Q. boissieri* was explained by physical factors in the backward selection technique. The most effective factor was the accumulated rain in the 3 weeks prior to sampling for ARQ_{ts} ($R^2 = 0.85$, $P < 0.001$) and for ARQ_{ts24} ($R^2 = 0.93$, $P < 0.001$). Soil moisture was also correlated with ARQ_{ts} ($R^2 = 0.71$, $P = 0.04$) and with ARQ_{ts24} ($R^2 = 0.82$, $P = 0.03$). One outlier point was excluded in each correlation. For the *Q. calliprinos* no

correlation was found. Inverse correlation with marginal significance was found between the *Q. boissieri* ARQ_{ts} at 20 cm above the ground and ARQ_{sa} ($R^2 = 0.41$, $P = 0.06$) after excluding 1 outlier point out of 10 (measured in April 2017 when ARQ_{ts} was minimal, Fig. 3).

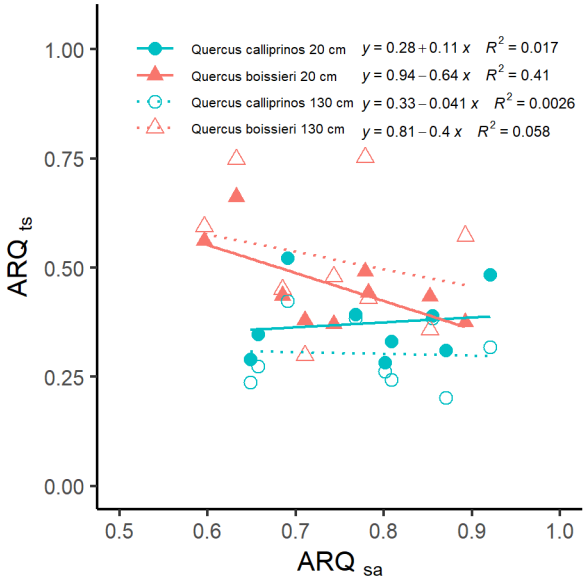


Figure 7. Scatter plot of stem ARQ (ratio of CO2 efflux/O2 influx) measured from incubated stem cores containing phloem and cambium tissues (ARQ_{ts}) sampled 20 (filled symbols) and 130 cm (hollow symbols) above ground from the main trunks of the tree species *Quercus calliprinos* and *Quercus boissieri,* against soil air ARQ measured below the same trees. Each point represents the mean value of three trees (stems and underlying soils) measured in each campaign. The *P* values of the correlations are 0.7393, 0.0618, 0.8973, and 0.533 ordered as appears in the legend.

4 Discussion

In the first part of the discussion we interpret the results of the current study. The second part is dedicated to discussion about methodology and future research towards more meaningful use of ARQ in soil and ecosystem studies.

4.1 Bulk-soil ARQ results indicate the respiratory substrate is more reduced than mean SOC

The overall weighted mean of the dissolution-corrected ARQ_{bs} is 0.75, well within the range of previous ARQ_{bs} assessments (Fig. 1) and similar to values of 0.75-0.80 measured in different natural soils in Germany [*Dilly*, 2001; 2003; *Dilly and Zyakun*, 2008]. An ARQ_{bs} value of 0.75 is appreciably below 0.95, the ARQ_{bs} expected by SOM-C_{ox} median value in soils meta-analysis study, and slightly below 0.77, the lowest expected value [*Worrall et al.*, 2013]. Several potential processes can be ruled out as drivers for the low weighted mean ARQ_{bs}, including dissolution of respired CO_2 in the soil water (after the dissolution correction) and reversible effects such O_2 uptake by oxidizing Fe^{2+} or Mn^{2+} that should be canceled by anaerobic respiration that must occur at different time in the soil (e.g. to reduce Fe and Mn). However, other processes with potentially irreversible effects, including microbial re-fixation of respired CO_2 and oxidative depolymerization are both possible explanations.

625 Studies of CO_2 'dark' fixation showed that rates scaled with microbial biomass regardless
626 of soil depth [*Akinyede et al.*, 2020]; thus we speculate that a constant fraction of respired CO_2 is
627 re-fixed regardless the season. If the re-fixation products, which are usually relatively oxidized,
628 are mineralized shortly after synthesis, no net effect on ARQ is to be expected. However, if the
629 re-fixed C persists in microbial biomass or SOM, there will be a net decrease in ARQ.
630 Nonetheless, the effect is small and accounting for the highest observed re-fixation rates (5.6%;
631 [*Akinyede et al.*, 2020]), only slightly increases our ARQ_{bs} mean, to 0.79.

632 Oxidative depolymerization breaks down macromolecules by introduction of oxygen-rich
633 groups, which forms small molecules that microbes can uptake easily. The polymer oxidation
634 step consumes O_2 and is expected to lower ARQ_{bs}. However, the mineralization of the highly
635 oxygenated produced molecules is expected to have high ARQ_{bs}, with overall a zero net effect on
636 ARQ_{bs}. Net reduction of ARQ_{bs} might occur if the oxygen-rich molecules escape microbial
637 digestion and associate with minerals, where an extended time period may pass until their
638 mineralization [*Kleber et al.*, 2015]. We estimate that in natural soils the fraction of C
639 associating to mineral surface is very small comparing to overall mineralization rates, thus the
640 net effect of oxidative depolymerization on ARQ_{bs} should be small. We therefore conclude the
641 mean ARQ_{bs} of 0.75 mainly reflects the stoichiometry of the respiratory substrate.

642 The difference between the expected ARQ_{bs} value according to the apparent SOM-C_{ox}
643 and the measured ARQ_{bs} suggests that the overall SOM is more oxidized than the smaller SOM
644 pool that is more accessible to microbes and directly supports respiration. Since oxidized
645 compounds are energetically more labile than reduced compounds (Table 1) the plethora of low
646 ARQ_{bs} results indicate that C_{ox} alone is insufficient to predict the decomposability of soil
647 compounds. The C_{ox} difference between the respiratory substrate and the total SOM is similar to
648 radiocarbon measurements where bulk-soil respired CO_2 is much younger than the total SOM
649 [*Trumbore*, 2000], which suggests most SOM has slow turnover rates with small contribution to
650 respiration. Hence, rather than representing the total SOM stoichiometry, ARQ_{bs} provides
651 information about the C_{ox} of the actual labile pool utilized by microbes.

652 ARQ_{bs} values lower than expected based on the bulk-SOM composition also indicates an
653 oxidation of the remaining, unrespired SOM (unless the C influx to the SOM has exactly same
654 C_{ox} as the respired C). This consequence is expected regardless the reason for the low ARQ_{bs}. If
655 reduced compounds with smaller O content are decomposed preferentially, the remainder SOM
656 is becoming more enriched with oxidized molecules. Same net effect is expected if the low
657 ARQ_{bs} is due to excess of bulk-soil O_2 uptake. This expectation is in line with observed SOM
658 oxidation trends over longer time scales. For example, Rock-Eval indices show increases in C_{ox}
659 (higher oxygen index and lower hydrogen index) with soil depth [*Sebag et al.*, 2016], with aging
660 of bare fallow [*Barré et al.*, 2016], and with experimental soil warming [*Poeplau et al.*, 2019].
661 Free-air CO_2 enrichment experiment (FACE) experiments also resulted in increased C_{ox} values in
662 SOM after nine years of elevated CO_2 treatment [*Hockaday et al.*, 2015]. The oxidation was
663 explained by a loss of reduced lipids and lignin and increased abundance of oxidized carbonyl
664 groups. Using the change in the C stocks, the authors further calculated the net respiration fluxes
665 accompanying the C_{ox} change (OR = 1.477), would be equivalent to an ARQ of 0.68, which is
666 similar to our mean ARQ_{bs} value of 0.75.

4.2 Fe^{2+} redox changes affect ARQ_{bs} only at low respiration rates

ARQ_{bs} values were associated with respiration (i.e. O_2 uptake) rate, which varied by two orders of magnitude in our experiments (Fig. 4, 5a). At respiration rates above ~3 nanomole O_2 $g.DW^{-1}$ min^{-1} most ARQ_{bs} values were similar to the mean weighted seasonal value (0.7-0.8) with relatively small variability, while during lower respiration rates larger ARQ_{bs} variability were observed (0.4-0.9; Fig. 4). One possible explanation for this observation is greater sensitivity of the O_2 measurement for smaller O_2 fluxes. However, the low ARQ_{bs} values measured at low temperatures (Fig. 3) and in the soil drying experiment (Fig. 5a) seem like part of a trend and not just arbitrary analytical noise. Alternatively, the ARQ_{bs} and O_2 uptake relation can be explained by a shift in the dominant processes. When respiration rates are low, slower processes (i.e. those normally contributing only in a minor way to larger O_2 fluxes), such as oxidative depolymerization and redox related changes in Fe, can have a measurable effect on ARQ_{bs}. As respiration rates increase the ARQ_{bs} value related to substrate-stoichiometry will drown out other effects.

In the jar incubations with soils recovering from anaerobic conditions ($[O_2]$ ~1%) the ARQ_{bs} was 0.37 and $[Fe^{2+}]$ decreased sharply. The stoichiometry for the overall oxidation of Fe^{2+} ions by O_2, $O_{2(aq)} + 4Fe^{2+} + 6H_2O \leftrightarrow 4FeOOH_{(s)} + 8H^+$ [*Burke and Banwart*, 2002], explains 27% of the drop in $[O_2]$, another third of the O_2 uptake can be explained by faster oxidation of soil organic matter that usually follows anaerobic conditions (e.g. [*Keiluweit et al.*, 2017]), while the last third can be explained by the same microbial respiration as in the control soils. For comparison, the initial $[O_2]$ in the control jars was ~7% and the subsequent ARQ_{bs} was 0.74 with no change in $[Fe^{2+}]$. As the air in the anaerobic treatment was nearly anoxic, the ARQ_{bs} difference between 0.74 and 0.37 seems to represent the maximal effect of Fe^{2+} oxidation for the site. However, the soil drying-rewetting experiment indicates Fe redox change has only a small influence under field conditions (Fig. 5). The decrease in ARQ_{bs} values on the 11^{th} day after soil wetting seems to be the result of Fe^{2+} oxidation that occurred around the same time – by this time point, respiration rates were already low. We estimated that the amount of O_2 decrease due to Fe^{2+} oxidation, which is equivalent to the amount of alternative oxidants during anaerobic respiration, is less than 10% of the O_2 flux when respiration rates were higher. In addition, the minimal $[O_2]$ measured in the soil air was 18.14% therefore anoxia might present only in microsites and not in the whole soil profile. Moreover, the fast $[O_2]$ transition from ~1% to 20.95% in the jar incubations that resulted the sharp ARQ_{bs} decrease is not expected in the field. Gradual $[O_2]$ increase with smaller effect on ARQ_{bs} is rather expected. Thus, we conclude that redox-related O_2 consumption is significant at Odem forest only at low respiration rates.

4.3 Seasonality of soil air ARQ indicates soil respiration is controlled by bulk-soil respiration

The overall mean ARQ_{sa} was 0.76, within the range of previous studies (Fig. 1). ARQ_{sa} was almost always higher than ARQ_{bs} and when ARQ_{root} was measured its values exceeded ARQ_{sa}, apparently confirming that ARQ_{sa} value is the weighted mean of those two end members and that ARQ can be used for partitioning of soil respiration sources (Fig. 6). Our results further support the view that variability in ARQ_{sa} reflects shifting dominance between root and microbially-mediated heterotrophic respiration sources [*Hicks Pries et al.*, 2019].

The backward selection technique indicates that on a yearly basis water-related parameters are the main factors controlling the seasonal ARQ_{sa} variability in this Mediterranean

site, with negligible effect of T_{soil} (Fig. 2a; Eq. 7). With two orders of magnitude variability, the bulk-soil O_2 uptake rate (at room temperature) was also mainly controlled by soil moisture (Fig 2d; Eq. 6). We lack information about the seasonal variability of root respiration rates, but based on other studies in Mediterranean climates, it can be assumed the rate from tree roots is rather constant [*Tang and Baldocchi*, 2005; *Vose and Ryan*, 2002]. We therefore estimate that in Odem forest ARQ_{sa} is mainly controlled by variability in ARQ_{bs}, which in turn mainly controlled by soil moisture. T_{soil} was important factor with positive effect on ARQ_{sa} only outside the high growth period (May to January) when soil respiration rates were slow (Fig. 2). A similar positive effect of T_{soil} on ARQ_{sa} was observed at 30 cm depth in heated soils (+4^{o}C) during winter [*Hicks Pries et al.*, 2019]. This trend is in accordance with the effect of temperature on ARQ_{bs}; a positive effect when respiration rates are slow, and no effect when respiration rates are high (Fig. 3, 4).

Deviations of ARQ_{sa} from ARQ_{bs} and ARQ_{root} values might resulted from soil profile processes that might be present in the field and not in the ARQ_{bs} and ARQ_{root} incubations. For example, CO_2 that otherwise would be released to the soil air might dissolve in the soil water and leach. This process can explain the few ARQ_{sa} values that were equal to or lower than ARQ_{bs} during the second and wetter winter (Fig. 2, 6). In contrast if the soil water does not leach the dissolved CO_2 can degasses back to the pore space after water evaporation or warming that reduces CO_2 solubility. Such degassing could explain the spike in ARQ_{sa} observed during the last campaign of the second winter (Fig 6). In the area of Odem forest only 10-30% of annual precipitation (950 mm) leaches to groundwater [*Dafny et al.*, 2006], most of it during episodic intensive rain events during winter. Therefore we estimate the loss of respired CO_2 to groundwater is negligible on an annual basis, though it might affect specific sampling dates.

4.4 Tree stems ARQ

The ARQ values measured in tree stem tissues were considerably lower than 1.0, the value expected from carbohydrate respiration (Table 1) in most plants [*Hoch et al.*, 2003; *Plaxton and Podestá*, 2007]. The mean ARQ_{ts} and ARQ_{ts24} values were 0.39 and 0.68, respectively. Damage during the tissue extraction from the stems might result in a burst of O_2 uptake that lowered ARQ_{ts} values. One indication for such a short-term wound response on O_2 uptake is elevated H_2O_2 production observed within two hours after epicormic shoot wounding [*Tian et al.*, 2015]. Therefore, ARQ_{ts24} (measured 24 h after tissues extraction) can be considered as less prone to artifacts, although metabolic change during the 24 h period is possible [*Hilman et al.*, 2019]. The ARQ_{ts24} mean values of ~0.7 could be explained by pure lipid substrates for respiration (Table 1). This is, however, not expected, especially in the tree genera *Quercus* [*Hoch et al.*, 2003; *Sinnott*, 1918]. The current results thus reinforce our recent studies and suggest that tree stem ARQ do not merely reflect the substrate's stoichiometry and additional non-respiratory effects are at work [*Angert et al.*, 2012; *Hilman et al.*, 2019].

Our observations indicate differences between the species that are related to phenology. During the foliated period of the deciduous *Q. boissieri* its ARQ_{ts} was similar to the evergreen *Q. calliprinos* values, however during winter exfoliation the *Q. boissieri* values increased in ~0.2 ARQ units above the *Q. calliprinos* values (Fig. 2). The seasonal change of ARQ_{ts} and ARQ_{ts24} of the *Q. boissieri* were explained by water related factors suggesting low tree stem ARQ is associated with dry conditions. We speculated that if re-fixation of respired CO_2 by the enzyme PEPC is the process that lowers tree-stems ARQ, the enzyme's products like the organic acids

citrate and malate might be exported via the phloem to the roots and be secreted to the soil as root exudates [*Hoffland et al.*, 2006; *Shane et al.*, 2004]. In agreement, a recent study in a different Mediterranean forest in Israel reported that root exudation rates were highest in the dry season and were associated with high soil temperature and low soil moisture [*Jakoby et al.*, 2020]. Indeed, the inverse relationship between ARQ_{ts} vs. ARQ_{sa} at 20 cm in the deciduous *Q. boissieri* (Fig. 7) might suggest catabolism of organic acids, with high ARQ in the soil offsetting low stem ARQ (Table 1). However the *Q. calliprinos* ARQ_{ts} values were rather uniform throughout the year without correlation with ARQ_{sa}. The fact that ARQ_{sa} of both species had almost the same seasonal changes suggests that the variability in ARQ_{sa} is not related to ARQ_{ts}. It is highly probable that our tube sampling from depth of ~15 cm, used to estimate ARQ_{sa}, did not fully capture the autotrophic signal of shallow fine roots. Section 4.5.2 adds more discussion about this topic.

4.5 What should be done next?

In this section we discuss future research of ARQ in soils. Suggested future steps in tree stem ARQ research are discussed at [*Hilman et al.*, 2019].

4.5.1 Bulk-soil incubations

For estimating ARQ_{bs} we measured the change of $[CO_2]$ and $[O_2]$ in the headspace of closed-system incubations. We further corrected for CO_2 dissolution in the soil moisture, especially due to the relatively basic pH value (6.6) and large amount of soil water per headspace volume. A way to reduce the amount of dissolved CO_2 is by increasing the incubation set-up. With fixed soil water content, increasing the ratio incubation headspace/ soil mass by a given factor would decrease the amount of dissolved CO_2 by the same factor.

We conclude that when respiration rates are high, the ARQ_{bs} signal mostly reflects the substrate stoichiometry of microbial respiration. One way to test this would be to amend soil incubations with compounds differing in their expected ARQ, for example lipids or soluble amino acids with ARQ < 1, sugars with ARQ = 1, and organic-acids with ARQ >1. Ideally the compounds should have different $\delta^{13}C$ signatures than the SOM to allow calculation of the contribution of the amended compound to the respired C. Previously, addition of sugars (with ARQ = 1) to bulk-soil incubations indeed resulted in higher ARQ_{bs} compared to basal values of 0.8, but the resulting ARQ values varied between 0.95 and 1.6 and depended on the added amount of sugar and the sampling timing after the amendment [*Dilly and Zyakun*, 2008]. High amounts of added substrate caused a 'priming effect' that rapidly altered microbial composition and metabolism, thus the resulting ARQ does not represent purely the stoichiometry of the amendment. We therefore suggest amending soils with smaller amounts of ^{13}C-labeled substrate to better mimic natural input rates. The role of oxidative depolymerization and CO_2 re-fixation in $ARQ_{\mathbf{bs}}$ variability can be tested by parallel measurement of ARQ_{bs} and the activity of oxidases (enzymes that use O to break-down large molecules) and rates of CO_2 re-fixation.

Temperature was found to have an effect on ARQ_{bs}. Moisture effects were not tested separately, but the higher ARQ_{bs} at 22 and 30°C in *Q. calliprinos* compared to *Q. boissieri* soils may reflect the higher soil moisture in the *Q. calliprinos* (Fig. 3). Higher water content can affect ARQ_{bs} via faster respiration (similarly to the discussed temperature effect), or via faster diffusion of dissolved organic C that can fuel microbial respiration with different substrates. Better understanding of these effects can also improve ARQ_{bs} predictions with seasonal changes.

Therefore factorial incubations in different temperatures and soil moistures to test these effects separately are recommended.

4.5.2 Total soil ARQ and portioning respiration sources

As mentioned in the introduction, the very few available soil-chamber-ARQ_{sa} measurements yielded higher values than estimated from gas profiles and diffusion modeling. The assumed reason is that the soil chamber better captures the signal of processes like respiration from fine roots (with high ARQ) that are usually concentrated in soil horizons above the ones sampled by gas probes (e.g. [*Claus and George*, 2005]). Under-representation of the shallow roots might explain the lack of the expected inverse stem-soil respiration ARQ relations. The influence of surface litter decomposition is another process not represented in gas diffusion models. Hence, to evaluate the contribution of soil respiration ARQ to the total ecosystems ARQ balance requires surface chamber measurements to at least compare with gas tube/diffusion estimates. However, measurement of ARQ using tube sampling is easier than chamber sampling since the O_2 signals are larger and easier to detect, and the sampling is fast and simple technically. Comparing both measurements is expected to yield interesting results, especially testing the assumptions that are presented in Section 2.3 and Eq. 1, and to make a sensitivity test to find under which conditions (e.g. advective mixing) Eq. 1 becomes invalid.

ARQ has the potential to partition the sources of soil respiration. In comparison to other non-destructive methods like the bomb-radiocarbon approach and canopy $\delta^{13}C$-labeling the use of ARQ can be technically simpler and more cost-effective [*Kuzyakov*, 2006]. There are few considerations that yet should be taken: different ARQ_{bs} signals from soils sampled from different depths, their temporal variability, and their relative contributions to the total respiration fluxes.

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