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Short Communication

Extraction of chlorophyll *a* from biological soil crusts: A comparison of solvents for spectrophotometric determination

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ABSTRACT

We tested the efficacy of four different commonly used solvents (acetone, ethanol, dimethyl sulfoxide, methanol) for the extraction of chl*a* from biological soil crusts of three different successional stages (dark, intermediate, and light). Our results indicate that a double extraction technique is necessary in order to achieve chl*a* recovery in the range of 76–87 percent. For all crust types, ethanol and dimethyl sulfoxide extracted the greatest amount of chl*a* using a two-extraction efficiency calculation.

Biological soil crusts are diverse microbial associations of cyanobacteria, fungi, lichen, and mosses, colonizing the top several millimeters of the soil surface in many arid and semi-arid ecosystems (West, 1990; Garcia-Pichel and Belnap, 1996). Soil crusts typically vary in coloration or level of development (Belnap et al., 2008) depending on crust successional stage. For the Colorado Plateau region, USA, late succession dark crusts contain greater chlorophyll *a* (chl*a*) concentrations owing to high cyanobacterial biomass and darker pigmented cyanobacterial species compared to early succession light crusts (e.g. Yeager et al., 2004). Thus, chl*a* concentration is a useful quantitative indicator of both the presence of autotrophs and degree of soil crust development. Because biological soil crusts are composed of a range of different chlorophyll containing organisms, finding an extraction method that efficiently and accurately estimates the chl*a* content in both early succession and late succession crusts is important to comparative studies. Moreover, soil crusts are composed of cyanobacteria and lichens inhabiting the unique conditions of an aridland soil matrix, and extraction of chl*a* from these organisms is likely to be very different than from organisms found in any other environment.

Spectrophotometric determinations of chl*a* concentrations have been successfully attempted for cyanobacterial organisms derived from a variety of environmental samples including: natural waters (e.g. Jeffrey and Humphrey, 1975), epilithic biofilms (Thompson et al., 1999), sedimentary rock (Bell and Sommerfeld, 1987), and soils (e.g. Beymer and Klopatek, 1991; Belnap et al., 1993; Hawkes and Flechtner, 2002). A wide array of solvents has been previously explored in the literature for the extraction phototrophic organisms. Researchers have noted that solvents can vary in their ability to extract chl*a* from different taxa (Pápista et al., 2002; Stich and Brinker, 2005), but because the risk of using a solvent with low extraction efficiency is low accuracy in terms of a determining actual chl*a* concentration, it is important to determine the most effective solvent for a particular set of samples. Studies comparing across more than one solvent have proposed the use of 100% methanol for intertidal rock biofilms (Thompson et al., 1999) and freshwater algae (Pápista et al., 2002) and researchers have favored DMSO over acetone for the extraction of epilithic microbial communities on sandstone (Bell and Sommerfeld, 1987). To our knowledge, however, there are no studies that formally compare the efficiency of multiple extraction solvents (acetone, ethanol, methanol, DMSO) for the spectrophotometric analysis of biological soil crust chl*a* to the same degree that we have attempted in this study.

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Soil crust collection took place during the Fall of 2009 from Canyon Rims Recreation Area, Utah (109°54′23″W; 38°0′12″N). Soils here are predominantly of the Rizno-Rock outcrop complex, characteristic of alluvial fine sandy loams with ~15% carbonate content (NRCS, UT633) derived from Cedar Mesa sandstone. We collected bulk soil crust samples (0–2 cm) by inserting a flat spatula into the soil profile and removing the dry crust surface. Three different levels of development (dark, intermediate, light), which were based on ocular estimates of crust color. In Canyonlands the poorly developed, light crusts are >90% dominated by the free-living, filamentous cyanobacteria, *Microcoleus vaginatus* (Yeager et al., 2004). The darker, older and more developed soil crusts tend to be dominated by *M. vaginatus* (>50%) but also contain *Microcoleus steenstrupii* (~17%), darker pigmented cyanobacteria *Scytonema myochrous* and *Nostoc commune* (~4%), as well as mosses (*Sytrichia caninervis*) and lichens (*Collema tenax* and *Collema coccophorum* groups) (~14%) (Yeager et al., 2004). Following collection, crusts were air dried, sieved (2 mm sieve) and ground to a fine powder with mortar and pestle. Replicate samples ($n = 6$) of each level of development (light, intermediate, dark) were weighed (3 g) into a screw-cap vial along with either 6 or 9 mL of solvent (Table 1). Alcohol and acetone solvents were neutralized with a small amount of $MgCO_3$ and filtered. Samples were placed on an orbital shaker for the appropriate extraction time. Heated samples (see Table 1) were cooled for at least 30 min prior to analysis. Samples were centrifuged and analyzed by spectrophotometer (Beckman DU-64, Beckman Instruments Inc., Fullerton, CA) immediately following extraction. For each test, the spectrophotometer was calibrated to each relevant wavelength with the appropriate extraction solution. Some calculations required sample acidification followed by a second measurement of absorbance after 90 s. Procedures requiring acidification are noted in Table 1. All sample processing was conducted in minimal light (HMSO, 1983) to avoid the degradation of chlorophyll.

We performed initial extractions testing extraction temperature, extraction time, and agitation. From our preliminary investigation, we chose the procedure that was most consistent and acquired the most chl*a* for each solvent and proceeded with more rigorous testing, wherein samples were extracted multiple times until there was no longer a detectable chl*a* signal. Most notable were the initial findings that cold acetone was a poor solvent compared to room temperature acetone and that methanol extracted the greatest amount of chla with a 5-h extraction compared to a 1 h or 18–24 h extraction. We initially found that agitation led to increased chl*a* extraction in all cases, with the exception of DMSO. For this reason later tests with DMSO were dispersed by hand rather than on the orbital shaker. In order to assess the effectiveness of extraction solvents, we calculated the combined efficiency of the first two extractions relative to the total chl*a* recovered. We found that extraction efficiency had to be normalized to the mean total chl*a* extracted by ethanol for each level of development, which had the highest recovery of chl*a* in each crust type (e.g. (μg chl*a* g soil^{-1} extraction 1 + μg chl*a* g soil^{-1} extraction 2)/total$_{ethanol}$ μg chl*a* g soil^{-1}).

We calculated chl*a* concentrations using methods and equations previously cited in the literature (Table 1). Ritchie (2006) did not examine DMSO, for this reason we calculated DMSO chl*a* values using the equation presented for acetone because there is precedent for this in the literature. Past researchers have calculated DMSO extracted chl*a* concentrations with extinction coefficients (e.g. Ronen and Galun, 1984) and calculations (APHA, 1980) that were developed for acetone. Though the Ritchie (2006) equation was not developed specifically for DMSO, employing it in this way gave us a reference to compare DMSO to other solvents. We display total chl*a* recovery data in Table 2 as calculated with multiple equations, but we chose to conduct statistical analyses on results in terms of the Ritchie (2006) equations only. The two-extraction efficiency presented below pertains to these equations as well. We chose to focus our formal evaluation on the Ritchie (2006) equations only because it provides a way to standardize our evaluation of different solvents while controlling for the source of the equations used to calculate chl*a*.

We used a two-factor analysis of variance (ANOVA) to examine the effect of crust type (dark, intermediate, light) and solvent type (acetone, ethanol, DMSO, methanol) on the amount of chl*a* extracted. Statistical analyses were completed with the PASW Statistics 18 package (SPSS). Tukey Highly Significant Differences (HSD) post-hoc tests were used to examine difference in the significance of ANOVA results.

Chl*a* was released during the first extraction step (39–71%), but also during subsequent extraction steps suggesting that multiple extractions are critical to obtaining appropriate estimates of soil crust chl*a* regardless of solvent (data not shown). Total chl*a* content of biological soil crust differed substantially by solvent ($F^*_{(3,60)} = 260.60$, $P < 0.000$), similar to the findings of Lan et al. (2011). In this study, the lowest amounts of chl*a* were recovered by acetone followed by methanol. As predicted, chl*a* content was higher in dark and intermediate crusts relative to light crusts ($F^*_{(2,60)} = 46.87$, $P < 0.000$). DMSO and ethanol extracted approximately the same amount of chl*a* (Table 2). However, there was

Table 1
Chl*a* equations and references for four solvents. For each equation, V = volume of solvent (mL), g soil = gram dry soil, L = path length. Equations account for both the degradation of chlorophyll *a* to phaeophytin and also for any turbidity in the sample by subtracting the sample absorbance at 750 nm from other absorbance values. Subscripts indicate absorbance before acid addition (e.g. Wavelength$_0$) or after acidification (Wavelength$_a$).

Reference	Temp.	Duration	Acidification	Calculation
90% Acetone	4 °C	24 h		
10200H (APHA, 1995)			X	$(26.7 \times (664_0 - 665_a) \times V)/(\text{g soil}^{-1}) \times L$
Ritchie (2006)				$(11.4062 \times (665_0) \times V)/(\text{g soil}^{-1}) \times L$
Ethanol	80 °C	5 min boil, 30 min cool		
10260 (ISO, 1992)			X	$(29.6 \times (665_0 - 665_a) \times V)/(\text{g soil}^{-1}) \times L$
Ritchie (2006)				$(11.9035 \times (665_0) \times V)/(\text{g soil}^{-1}) \times L$
DMSO	65 °C	45 min		
APHA (1980)			X	$(26.7 \times (665_0 - 665_a) \times V)/(\text{g soil}^{-1}) \times L$
Ritchie (2006)				$(11.4062 \times (665_0) \times V)/(\text{g soil}^{-1}) \times L$
Methanol	18 °C	5 h		
Porra et al. (1989)				$(16.29 \times 665_0 - 8.54 \times 652_0) \times V/(\text{g soil} - 1) \times L$
Ritchie (2006)				$((-8.0962 \times 652_0) + (16.5169 \times 665_0)) \times V/(\text{g soil} - 1) \times L$

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Table 2
Total chl*a* recovered (μg chl*a* g soil^{-1}). Data are presented as means with standard error in parentheses for all calculations. The number of successive extractions completed for each solvent is indicated. For all solvents ($n = 6$).

Solvent & Calculation	# Extractions	Crust type		
		Dark	Intermediate	Light
90% Acetone	5			
10200H (APHA, 1995)		7.67(0.10)	7.44(0.05)	1.80(0.14)
Ritchie (2006)		9.25(0.18)	8.16(0.22)	2.29(0.09)
Ethanol	5			
10260 (ISO, 1992)		13.79(0.18)	14.05(0.22)	5.02(0.12)
Ritchie (2006)		12.37(0.31)	12.41(0.18)	4.99(0.11)
DMSO	4			
APHA (1980)		8.23(0.18)	8.50(0.14)	3.49(0.11)
Ritchie (2006)		11.26(0.25)	10.85(0.23)	4.08(0.08)
Methanol	3			
Porra et al. (1989)		8.94(0.04)	9.70(0.15)	3.50(0.07)
Ritchie (2006)		9.20(0.04)	9.98(0.15)	3.58(0.07)

a significant interaction between solvent and crust color ($F^*_{(6,60)} = 10.78$, $P < 0.000$) owing to the fact that acetone extracted the least amount of chl*a* from intermediate and light, but not dark crusts (Table 2). Extraction efficiency of the first two extractions (normalized to the mean total chl*a* recovered by ethanol for each level of development) ranged from 79 to 82% for DMSO and 75 to 87% for ethanol (Fig. 1a,b,c).

Care should be taken to identify factors such as toxicity of solvents, expense, number of extractions, and efficiency across different levels of development, when choosing a solvent/methodology for this type of routine extraction. Acetone is commonly used for chlorophyll extraction and is the most widely utilized solvent in the literature (e.g. Jeffrey and Humphrey, 1975). However, our results show that acetone has low extraction capabilities in these biological soil crusts, a result which others have observed with respect to many phototrophic organisms (Ritchie, 2006, and references within), and we suggest that it should not be used in the extraction of chl*a* from soil crust. Methanol has been reported to have high extraction efficiency with respect to recalcitrant organisms (e.g. Holm-Hansen and Riemann, 1978; Thompson et al., 1999; Ritchie, 2008), and in our tests we found that it did a sufficient job at extracting chl*a* with the same efficiency across different levels of development (Fig. 1). However, with methanol at least three extractions were necessary to capture >75% of chl*a*. Past researchers have noted with methanol, there is low analytical resolution when using spectrophotometry and that it may cause some chlorophyll degradation over long extraction times, and both of these reasons have deterred its widespread use as a solvent. More recent investigations have provided support for the improved chromatographic resolution when buffered with ammonium acetate (Wright et al., 1997) or $MgCO_3$, as in this study. DMSO is a solvent that has been used by previous researchers in the extraction of biological soil crust chl*a* (Beymer and Klopatek, 1991; Belnap et al., 1993; Hawkes and Flechtner, 2002) as well as lichens (Ronen and Galun, 1984). In our tests, we found that DMSO is well suited for the extraction of all soil crusts (Fig. 1), but there are significant health concerns associated with the routine use of DMSO that should be taken into consideration. Past studies have noted that hot ethanol preparations have high chl*a* extraction efficiencies for organisms that are difficult to extract (Ritchie, 2008), which is supported by this study and the finding that ethanol was one of the more efficient solvents tested here. Additionally, ethanol is relatively non-toxic, thus making it a more desirable solvent to work with in laboratory extractions.

We conclude that the extraction of chl*a* from biological soil crust is possible with a range of different solvents, however in this study we observed that ethanol and DMSO extracted chl*a* most efficiently in terms of a two-extraction procedure. Moreover, ethanol extracted the greatest total amount of chlorophyll from all crust types. This study points to important considerations for the extraction of chl*a* from an aridland soil matrix and we believe that the work presented here could be a starting point for a methodological standardization. It is important to point out that we limited our formal analysis to a specific set of calculations that were simultaneously developed for multiple solvents (Ritchie, 2006), however,

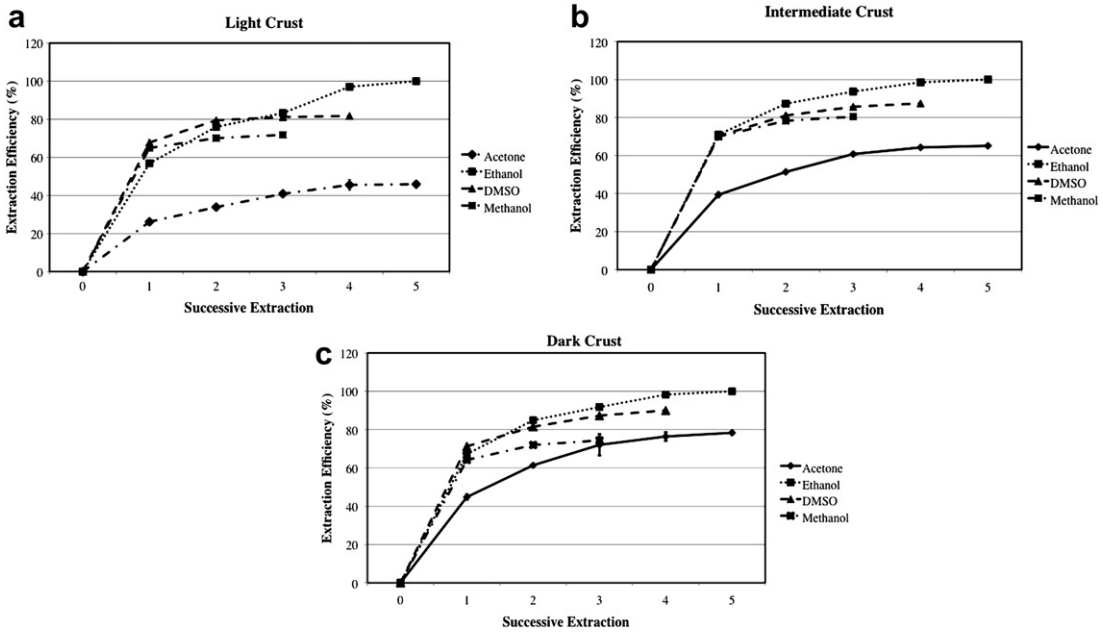

Fig. 1. Extraction efficiencies for light (a), intermediate (b) and dark (c) biological soil crust. Extraction efficiencies are calculated relative to Ethanol, which overall recovered the greatest total chl*a* of all solvents. Data represent calculations of chl*a* based on the equations presented by Ritchie (2006) only.

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of across all of our calculated results it is obvious that there are major discrepancies between equations for each solvent (Table 2). Future work should include the adoption of a common set of equations for calculation chl*a* content.

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References

APHA, 1980. Standard methods for the Examination of water and Waste water: standard method 10260. American Public Health Association, Washington, DC.

APHA, 1995. Standard methods for the Examination of water and Waste water: standard method 10200H. American Public Health Association, Washington, DC.

Bell, R., Sommerfeld, M., 1987. Algal biomass and primary production within a temperate zone sandstone. American Journal of Botany 74, 294–297.

Belnap, J., Harper, K., Warren, S., 1993. Surface disturbance of cryptobiotic soil crusts: nitrogenase activity, chlorophyll content, and chlorophyll degradation. Arid Land Research and Management 8, 1–8.

Belnap, J., Phillips, S.L., Witwiki, D.L., Miller, M.E., 2008. Visually assessing the level of development and soil surface stability of cyanobacterially dominated biological soil crusts. Journal of Arid Environments 72, 1257–1264.

Beymer, R., Klopatek, J., 1991. Potential contribution of carbon by microphytic crusts in pinyon-juniper woodlands. Arid Land Research and Management 5, 187–198.

Garcia-Pichel, F., Belnap, J., 1996. Microenvironments and microscale productivity of cyanobacterial desert crusts. Journal of Phycology 32, 774–782.

Hawkes, C., Flechtner, V., 2002. Biological soil crusts in a xeric Florida shrubland: composition, abundance, and spatial heterogeneity of crusts with different disturbance histories. Microbial Ecology 43, 1–12.

HMSO, 1983. The Determination of Chlorophyll-a in Aquatic Environments. HMSO Publications, London.

Holm-Hansen, O., Riemann, B., 1978. Chlorophyll a determination: improvements in methodology. Oikos 30, 438–447.

ISO 10260, 1992. Water Quality, Measurement of Biochemical Parameters; Spectrometric Determination of the Chlorophyll-a Concentration. Beruth Verlag GmbH Berlin, Zurich.

Jeffrey, S., Humphrey, G., 1975. New spectrophotometric equation for determining chlorophyll a, b, c1 and c2. Biochemie und Physiologie der Pflanzen 167, 194–204.

Lan, S., Wu, L., Zhang, D., Hua, C., Liu, Y., 2011. Ethanol outperforms multiple solvents in the extraction of chlorophyll-a from biological soil crusts. Soil Biology and Biochemistry. doi:10.1016/j.soilbio.2010.12.007.

Pápista, É, Acs, É, Böddi, B., 2002. Chlorophyll-a determination with ethanol – a critical test. Hydrobiologia 485, 191–198.

Porra, R.J., Thompson, W.A., Kriedemann, P.E., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta 975, 384–394.

Ritchie, R., 2006. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynthesis Research 89, 27–41.

Ritchie, R., 2008. Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol. Photosynthetica 46, 115–126.

Ronen, R., Galun, M., 1984. Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation. Environmental and Experimental Botany 24, 239–245.

Stich, H., Brinker, A., 2005. Less is better: uncorrected versus pheopigment-corrected photometric chlorophyll-a estimation. Archiv für Hydrobiologie 162, 111–120.

Thompson, R., Tobin, M., Hawkins, S.J., Norton, T.A., 1999. Problems in extraction and spectrophotometric determination of chlorophyll from epilithic microbial biofilms: towards a standard method. Journal of the Marine Biological Association of the United Kingdom 79, 551–558.

West, N., 1990. Structure and function of microphytic soil crusts in wildland ecosystems of arid to semi-arid regions. In: Begon, M., Fitter, A.H., Macfadyen, A. (Eds.), Advances in Ecological Research, vol. 20. Academic Press Inc., San Diego, pp. 179–281.

Wright, S., Jeffrey, S., Mantoura, R., 1997. Evaluation of methods and solvents for pigment extraction. In: Jeffrey, S., Mantoura, R., Wright, S. (Eds.), Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods. UNESCO, Paris, pp. 261–282.

Yeager, C., Kornosky, J., Housman, D.C., Grote, E.E., Belnap, J., Kuske, C.R., 2004. Diazotrophic community structure and function in two successional stages of biological soil crusts from the Colorado plateau and Chihuahuan desert. Applied and Environmental Microbiology 70, 973–983.

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