HortScience 41(3):815–817. 2006.

Dissimilatory Reduction of Sulfate in Black Layer

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Additional index words. creeping bentgrass, redox potential, sulfate-reducing bacteria, sulfide, sulfur

Abstract. **Black layer has been associated with a severe decline in the quality of turf on putting greens. It was suggested that the black layer results from dissimilatory sulfate (SO_4^{2-}) reduction. This study was done to determine if SO_4^{2-} reduction occurs in an existing black layer. Radioactive $^{35}SO_4^{2-}$ was used to calculate the rate of SO_4^{2-} reduction in intact soil cores taken from an existing black layer in a 'Penncross' creeping bentgrass (*Agrostis palustris* Huds. 'Penncross') putting green. When 10^{-3} *M* $^{35}SO_4^{2-}$ with a specific activity of 1.554×10^5 Bq·mg^{-1} SO_4^{2-} was injected into a core it reduced to sulfide ($^{35}S^{2-}$) at a mean rate of 7.1 nmol sulfur (S)/cm^3 soil/d. Injecting azide (N_3^-) or molybdate (MoO_4^{2-}) at 10% w/v with the label reduced the rate of SO_4^{2-} reduction to 0.03 and 0.01 nmol S/cm^3 soil/d, respectively. The effect of N_3^- confirmed that reduction of SO_4^{2-} was biological, while the effect of MoO_4^{2-} confirmed that the entities responsible for the reductive cycling were sulfate-reducing bacteria (SRBs). This was the first proof that biological reduction of SO_4^{2-} produces S^{2-} in a black layer from a creeping bentgrass putting green. It was concluded that the respiration of indigenous SRBs was linked to development of this black layer. Thus, a key to successfully controlling black layer in putting greens must involve regulating the respiratory activities of SRBs.**

Black layer is the term for a condition in which the rootzone soil in putting greens turns black. Sometimes the entire soil profile is blackened uniformly. Other times, a black band of variable thickness and location in the profile is observed. Alternatively, black flecks of variable size can also be observed. Regardless of style, a decline in the quality of the turf frequently accompanies the onset of black layer. Turf decline symptoms range from a bronzing and/or thinning of turf to loss, which impacts the functional quality and visual integrity of an affected green. Because of its impact on turfgrass quality, black layer was said to be the number one problem of creeping bentgrass (*Agrostis palustris* Huds.) greens in the late 1980s (Scott, 1986). It was also said that the origin, nature, and mechanism of its formation are not very well understood (Smiley et al., 1992).

Blackening of soil was attributed to the accrual of metal sulfides (MeS) such as manganese sulfide (MnS) or iron sulfide (FeS) (Berndt and Vargas, 1987; Rankin, 1988). Berndt (1990) spot tested the black layers from various golf greens for the presence of MeS using a solution of sodium azide (NaN_3) and iodine (I_2) as described by Fiegel (1972). All the samples analyzed tested positive for the presence of MeS. The black layers that were examined exhibited a foul odor and loss of the blackened condition with exposure to air. Some but not all were associated with severe turf decline. Experimental black layers created by adding elemental sulfur (S^0) to a waterlogged sand also tested positive for the presence of MeS (Berndt et al, 1987; Berndt, 1990). As with natural black layers, the experimental black layers were associated with a foul odor and loss of the blackened condition with exposure to air.

Metal sulfides readily form when hydrogen sulfide (HS^-) is released to the soil (Atlas and Bartha, 1980). For example, ∝-FeS (trolite) forms at $pe + pH = 5.63 + 0.12 \log SO_4^{2-} - 0.23$ pH (Lindsay, 1979) provided sufficient HS^- and iron II (Fe^{2+}) are available:

$HS^- \rightarrow H^+ + S^{2-}$ pK = 12.90

$S^{2-} + Fe^{2+} \rightarrow$ ∝-FeS pK = 16.21

There are two pathways by which HS^- becomes available in soils (Paul and Clark, 1996). They are the anaerobic mineralization of organic S and the dissimilatory reduction of inorganic S. The mineralization of organic S occurs via sulfatase enzymes occurring in fungi and bacteria. Dissimilatory reduction of inorganic S occurs via the respiratory activities of sulfate-reducing bacteria (SRBs) where compounds like SO_4^{2-} are used as terminal electron (e^-) acceptors.

It was assumed but never proven that the dissimilatory reduction of inorganic S was the pathway by which most black layers formed (Berndt and Vargas, 1987; Berndt et al., 1987). One reason for this was that when S^0 was applied to putting greens it was often linked to severe outbreaks of black layer. Incidents of severe black layer were also associated with high levels of SO_4^{2-} in irrigation water. Several other research reports (Burpee and Anderson, 1987; Gockel 1987; Goss, 1987; Rankin, 1988; Smith, 1988) professed but did not prove an association between the formation of black layer and dissimilatory SO_4^{2-} reduction. As early as 1974, suggestions were made linking the formation of black layer to the respiratory activities of SRBs like *Desulfovibrio* (Kozelnicky, 1987). Thus, the objective of the laboratory study described in this paper was to determine if dissimilatory SO_4^{2-} reduction could be verified in a naturally occurring black layer from the rootzone of a creeping bentgrass putting green.

Materials and Methods

Carrier-free $Na_2{}^{35}SO_4^{2-}$ (New England Nuclear) was used in this experiment to calculate rates of SO_4^{2-} reduction. The radioactive tracer was injected into intact cores taken from a black layer that was located in the rootzone of a creeping bentgrass putting green. The $^{35}S^{2-}$ that formed was distilled from the soil and quantified via liquid scintillation counting. From that data rates of SO_4^{2-} reduction were calculated.

Intact sample cores of black layer soil were collected from the rootzone of a 'Penncross' creeping bentgrass research putting green located at the Robert Hancock Turfgrass Research Center in East Lansing, MI. The rootzone was originally constructed as a modified soil (Beard, 1973) composed of 88.3% sand, 5.0% silt, and 6.7% clay as determined by the pipette method (Gee and Bauder, 1986). Soil cores had a moisture content of 10% by weight at the time of sampling as determined by gravimetry (Gardner, 1986). Air dried soil from 0 to 15 cm depth was 0.07% N as determined by Kjeldahl analysis (Bremner and Mulvaney, 1982) and 0.8% C as determined by dry combustion (Nelson and Sommers, 1982) yielding an average C:N of 11.4:1. The SO_4^{2-} concentration in air-dried soil (Tabatabai, 1974) averaged 33 µg SO_4^{2-}/cm^3 soil. Most probable number estimates of sulfate-reducing bacteria (Alexander, 1982) indicated an approximate concentration of between 3.3×10^3 and 7×10^5 SRBs/g soil.

The cores were collected by impaling plastic 10 cm^3 syringe barrels with the ends cut away horizontally into an existing band-type black layer that was located 10 cm deep in the rootzone. This black layer was composed of an unknown form of MeS. This was confirmed by spot testing with a solution of NaN_3-I_2 as described by Feigl (1972). By this test, MeS was present in soil if bubbling occurred upon introducing soil into the NaN_3-I_2 solution. Bubbling occurred because MeS catalyzes production of NaI, with the evolution of N_2 gas:

$2\ NaN_3 + I_2 \xrightarrow{MeS} 2\ NaI + 3\ N_2$

Black layer soil cores were then capped with rubber stoppers and stored in an atmosphere of 90:10 N_2:H_2 at ambient temperature in the dark until time for use. This was done to prevent oxidation of the MeS in the soil. Black layer was visible in each core used but was

Received for publication 7 Dec. 2005. Accepted for publication 23 Jan. 2006.
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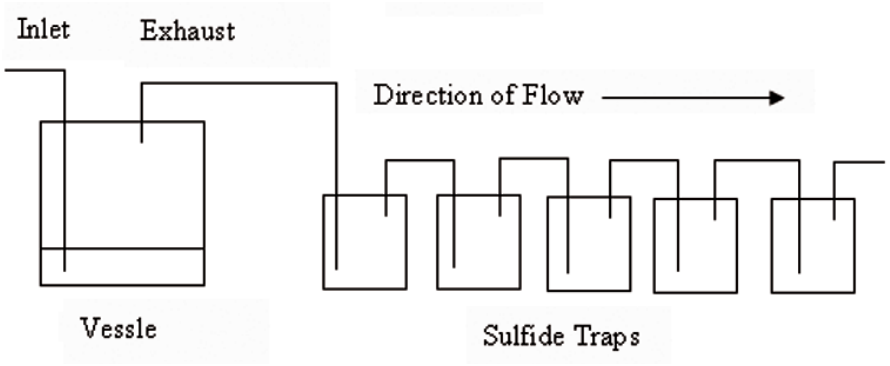

Fig. 1. Schematic design of the anaerobic S^{2-} still used to collect $^{35}S^{2-}$. Oxygen-free N_2 was used as the carrier gas, and 20-mL glass screw-top liquid scintillation vials containing 3 mL anoxic 2% $CdCl_2$ were used for the S^{2-} traps. The N_2 was deoxygenated by sparging over hot Cu^{2+} filings.

variable in appearance from core to core.

A tracer solution was prepared by introducing carrier-free $Na_2{}^{35}SO_4$ (New England Nuclear) into 10^{-3} *M* Na_2SO_4 to produce a 10^{-3} *M* stock solution of $Na_2{}^{35}SO_4$. The tracer solution had an activity of 11,100 Bq per 0.5 mL, which equated to a specific activity of 155,400 Bq per mg SO_4^{2-}.

Two control solutions were then prepared by amending portions of the stock tracer with either sodium azide (NaN_3) or sodium molybdate (Na_2MoO_4) to a final concentration of 10% w/v. The controls were used to prevent reductive cycling from occurring. Azide (N_3^-) is a nonselective biocide which prevents the occurrence of respiratory electron transport (Smith et al., 1983), while MoO_4^{2-} is a competitive inhibitor of sulfate-reducing bacteria that is not toxic to other soil organisms (Smith, 1981).

Purging and then subsequently recovering $^{35}S^{2-}$ from the soil was a basis for this study. To do so required the construction of an anaerobic still similar to the one described by Jorgensen and Fenchel (1974) and Smith (1981) (Fig. 1). The still consisted of a 200 mL wide-mouth screw top glass reaction vessel with a detachable lid. The lid was linked via port A to an upstream source of O_2-free N_2, and via port B to a downstream series of $^{35}S^{2-}$ traps. The traps were 20 mL glass scintillation vials containing 3 mL of anoxic 2% $CdCl_2$ connected in series via glass tubing. All line connections were made directly to vial caps so that the traps could be changed as required. The trapping train terminated into a 125 mL Erlenmeyer flask containing 100 mL 2% $CdCl_2$ to ensure complete recovery of all $^{35}S^{2-}$ during distillation. A third port in the lid was fitted with a rubber septum to facilitate entry into the reaction vessel as necessary.

For the experiment 11,100 Bq of stock tracer was injected into the center of each of 3 soil cores using a 1-cm^3 syringe fitted with a 3.8 cm 22 ga needle. Each of the cores was then sealed using rubber stoppers and incubated in an 90:10 atmosphere of N_2:H_2 at 23 °C in the dark for 48 h.

After the incubation period, one of the cores was removed from its syringe barrel and placed in the reaction vessel. The lid was attached, traps containing 3 mL of 2% $CdCl_2$ were installed, and then the vessel and trapping train were purged with O_2-free N_2 for 1 min. Next, 20 mL of freshly boiled distilled H_2O cooled to room temperature under an atmosphere of N_2 was added to the vessel to help disperse the soil core. The flow rate of the O_2-free N_2 was then adjusted to 100 $cm^3 \cdot min^{-1}$. This process was repeated for each sample core. This phase of the distillation process collected gaseous or water soluble $H^{35}S^-$.

After 30 min, the gas flow was halted, the first set of traps were removed, and then fresh traps were installed. Two milliliters of anoxic 37% HCl was then injected into the vessel to reduce solution pH to 1 to 2. The flow rate of the O_2-free N_2 was again adjusted to 100 $cm^3 \cdot min^{-1}$, and the distillation process was continued for another 30 min. Then, the gas flow was halted and the second set of traps was removed. This process was repeated for each sample core. This phase of the distillation process collected precipitated $^{35}S^{2-}$ that was volatile in acid.

For each $^{35}S^{2-}$ trap 15 mL of liquid scintillation counting (LSC) cocktail for aqueous solutions (RPI, Mt Prospect, Ill.) was added directly. The radioactivity distilled into each trap was then determined by counting in a Beckman (Fullerton, Calif.) LS 8100 scintillation counter. All samples were corrected for quenching by the H# method (Beckman, Fullerton, Calif.). Rates of SO_4^{2-} reduction were then calculated using the following formula from Sorokin, (1962):

$$\frac{([SO_4^{2-}])(a)(1.06)}{(A)(V)(t)} = \text{nmols S cm}^{-3}\ \text{d}^{-1}$$

where $[SO_4^{2-}]$ is the concentration of SO_4^{2-} in $nmol \cdot cm^{-3}$, (a) is the total number of µCi of $H_2{}^{35}S$ plus $Me^{35}S$, 1.06 is an isotope correction factor for the microbial fractionation of ^{32}S and ^{35}S isotopes used by both Jorgensen and Fenchel, (1974) and Sorokin, (1962), (A) is the original amount of radioactivity in µCi added, (V) is the soil volume in cm^3, and (t) is the incubation time. The mean residence time (T_{mrt}) of the SO_4^{2-} pool in the soil was calculated by dividing the initial $[SO_4^{2-}]$ by the calculated rate of SO_4^{2-} reduction. The residence half-life of the ^{35}S label (T_{rhl}) was calculated by the formula (A) ÷ (a)(2).

Experimental controls were included in the study by using the modified tracer solutions as described above. For the controls, 11,100 Bq of ^{35}S-N_3^-amended tracer was injected into the center of a set of three cores. Likewise, 11,100 Bq of ^{35}S-MoO_4^{2-}amended tracer was injected into the center of a different set of three cores. Control cores were then subjected to the incubation and the distillation/counting process as described above.

Collected data was analyzed statistically as a completely random design with three treatments replicated three times. Means were separated using Duncan's multiple range test ($P \leq 0.05$)

Results and Discussion

Dissimilatory reduction of $^{35}SO_4^{2-}$ occurred in the intact black layer at rates varying between 3 and 14 nmol S/cm^3 soil/d (Table 1.). As a result, $H^{35}S^-$ was released and an acid-volatile $^{35}S^{2-}$ fraction subsequently formed and accrued. About 32% of the injected $^{35}SO_4^{2-}$ reduced into $^{35}S^{2-}$. Between 80 to 95% of the $^{35}S^{2-}$ was in an acid-volatile fraction. This suggested that most of the HS^- produced in this soil during reductive cycling bonded with divalent metals such as Fe^{2+} or Mn^{2+} forming an acid-volatile MeS precipitate.

While most of the $H^{35}S^-$ probably bonded with soil metals about 5% to 15% was considered free. This implied that during reductive cycling some free HS^- is present at any given time. Free HS^- could be a cause of the turf decline associated with black layer. It is a cell poison that prevents the reduction of cytochromes a–a_3 during electron transport (Smith et al, 1983). This substance was shown to be highly toxic to creeping bentgrass (Berndt, 1990), and would likely be toxic to many kinds of rhizosphere organisms and animals.

The reduction of $^{35}SO_4^{2-}$ in this study was a biological process performed by SRBs. This was evidenced by the effects that both N_3^- and MoO_4^{2-} had on rates of reduction. Azide curtailed the rate of SO_4^{2-} reduction by suffocating soil organisms nonselectively. As with HS^-, the N_3^- prevents reduction of cytochromes a–a_3 during respiration (Smith, et al., 1983). Molybdate lowered the rate of SO_4^{2-} reduction because it competitively inhibited the activity of ATP-sulfurylase, found only in SRBs (Paul and Clark, 1996). The MoO_4^{2-} blocked the first step in the sulfate-reduction pathway without affecting the respiratory systems of other soil organisms (Smith, 1981).

There are several environmental conditions that must be satisfied before SO_4^{2-} is biologically reduced to HS^- in soil. First, an adequate population of SRBs must be present. Sulfate-reducing bacteria are anaerobic bacteria that are found in many soils over a wide range of environmental conditions (Paul and Clark, 1996). Dissimilatory SO_4^{2-} reduction is now recognized in a large number of bacterial genera including *Desulfovibrio*, *Desulfococcus*, *Desulfomonas*, and *Desulfobacter* (Paul and Clark, 1996). While specific species of SRBs were not determined population estimates in the experimental soil were between 3.3×10^3 to 7×10^5 SRBs/g soil. The indigenous population of SRBs in this black layer was apparently sufficient to reduce SO_4^{2-} at rates comparable to those calculated for marine sediments located in the Black Sea, the Bay of

Table 1. Mean rates of dissimilatory $^{35}SO_4^{2-}$ reduction in intact soil cores taken from the rootzone of a 'Penncross' creeping bentgrass putting green.

Treatment	S^{2-} Fraction[z]	Bq $^{35}S^{2-}$ Recovered	Rate[y]	T_{mrt}[x]	T_{rhl}[w]
$^{35}SO_4^{2-}$	$H^{35}S^-$	162.80 b[v]	7.10 a	71.1 c	2.1 c
	$Me^{35}S$	3,503.90 a			
$^{35}SO_4^{2-} + NaN_3$	$H^{35}S^-$	1.49 c	0.03 b	15,696.8 b	831.9 b
	$Me^{35}S$	5.14 c			
$^{35}SO_4^{2-} + Na_2MoO_4$	$H^{35}S^-$	0.81 c	0.01 c	39,870.0 a	2,090.3 a
	$Me^{35}S$	1.85 c			

[z]Sulfide fraction recovered as hydrogen sulfide ($H^{35}S$) or acid-volatile metal sulfide ($Me^{35}S$).
[y]Rate of dissimilatory SO_4^{2-} reduction in nmol S/cm^3/soil/d.
[x]Residence time of the SO_4^{2-} pool in days.
[w]Residence half-life of the $^{35}SO_4^{2-}$ label in days.
[v]Any two means within a column not followed by same letter are significantly different by Duncan's multiple range test at $P \leq 0.05$.

Keil, and the St. Barbara Basin (Jorgensen and Fenchel, 1974).

In addition to having an ample population of SRBs there must be an adequate supply of soil organic matter (OM) and SO_4^{2-} or some other form of inorganic S such as S^0 (Berndt and Vargas, 1992). The OM is food for the bacteria and is the ultimate source of the electrons that drive the reduction of SO_4^{2-}. Low molecular weight organic acids, alcohols, and molecular hydrogen (H_2) are also used as electron donors (Paul and Clark, 1996). In turn, inorganic S of some form is the electron acceptor for SRBs. If either OM or inorganic S is absent then SRB respiration does not occur, HS^- is not produced, and MeS does not form (Berndt, 1990; Berndt and Vargas, 1992).

Perhaps the most important condition for the occurrence of SO_4^{2-} reduction is a low redox potential (Connell and Patrick, 1968; Goldhaber and Kaplan 1975). Reduction of SO_4^{2-} to H_2S theoretically occurs at an $Eh' = -0.21$ v as calculated by Berndt and Vargas (1996):

$SO_4^{2-} + 4\ H_2 + 2\ H^+ \leftrightarrow H_2S + 4\ H_2O$

$\Delta G^{\prime}_r = +\ 9.61\ Eh' = -0.21$

$pe + pH = 5.21 - 1/8\ pSO_4^{2-} + 1/8\ pH_2S - 1/4\ pH$

Low redox potential is required for the process because most types of SRBs are obligate anaerobes that can be poisoned by traces of O_2 (Atlas and Bartha, 1981; Paul and Clark, 1996). The redox potential of the experimental soil was not measured, but the fact that MeS formed in the soil before the study was evidence that suggested it was relatively low. This should be considered unique as coarse textured, reasonably well drained terrestrial soils like those found in golf putting greens usually have a high enough redox potential to avert reduction of SO_4^{2-}.

Lowering of redox potential in greens could occur when rates of microbial respiration are high, when rates of O_2 diffusion are restricted, and/or when O_2 scavengers like S^0 or organic N are present (Berndt, 1990; Berndt and Vargas, 1992; Berndt and Vargas, 1996). It would seem logical, though, that each black layer situation should have its own set of particulars that are somehow related to low redox potential. For example, some black layers may develop as a result of the application of S^0 to depress pH (Berndt, et al., 1987; Berndt, 1990). Oxidation of applied S^0 represents an O_2 sink that can create a low redox potential in soil, especially if organic carbon of some type is plentiful (Berndt and Vargas, 1992). Application of S^0 also provides the soil with an abundance of inorganic molecules (i.e., S^0, SO_4^{2-}) that can function as e^- acceptors in reduction processes. Nitrification of applied organic N can lower redox potential by scavenging O_2 thus allowing HS^- to be released (Berndt, 1990). Alternatively, denitrification can also help to produce low redox conditions in turf soils by depleting NO_3^-, provided that the concentration of O_2 is also relatively low (Berndt, 1990; Berndt and Vargas, 1996). In other situations low redox conditions could result because of restricted O_2 diffusion resulting from water-logging (Berndt, 1990), an accumulation of surface algae(s) as suggested by Hodges (1989), the presence of biofilms as suggested by Cullimore et al. (1990), or the development of a surface salt crust or internal soil layers (Berndt, 1990). Turnover of algal cells and biofilm constituents would also constitute an excellent source of donor electrons for the sulfate-reduction process.

This research proved that dissimilatory reduction of SO_4^{2-} was an active pathway for the release of S^{2-} in an existing black layer within a creeping bentgrass putting green. This research also proved that entities responsible for the reductive cycling were SRBs of unknown genera. Since dissimilatory SO_4^{2-} reduction is a bacterial respiration process (Berndt, 1990; Berndt et al., 1987; Berndt and Vargas, 1992; Berndt and Vargas, 1996) the key to successfully controlling the release of S^{2-} in putting green root zones via this process must therefore involve controlling the respiratory activities of SRBs. One way this might be accomplished is by poising soil redox potential at a point high enough to avert release of S^{2-} by introducing alternate e^- acceptors such as NO_3^- (Berndt and Vargas, 1996), and by limiting the input of e^- acceptors such as S^0 and e^- donors such as organic C (Berndt and Vargas, 1992).

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