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LETTER TO THE EDITOR

NO news is no new news

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Abstract

In the paper 'NO News', Preston *et al.* (2004) make a number of erroneous assumptions regarding nitrogen oxide chemistry. These authors also present some very significant misinterpretations of previous research into the effects of various nitrogen oxides on germination of post-fire followers. Methodological differences between the study by Preston *et al.* (2004) and previous work are also problematic, such as using NO-donors in solution versus the use of direct application of various nitrogen oxides in the gaseous phase. A closer review of these studies, with the proper understanding of nitrogen oxide chemistry, and interpretations of the available literature, would lead to the conclusion that, contrary to the authors' assertions, the Preston *et al.* (2004) study supports, rather than refutes, earlier findings by Keeley and Fotheringham (1997, 1998a, b, 2000).

NO_x chemistry background

Nitrogen oxides have a complex chemistry, and the various nitrogen oxides have very different chemical properties. Preston *et al.* (2004) confuse the reader by treating nitrogen monoxide (nitric oxide, NO) and nitrogen dioxide (NO_2) as if they were interchangeable, and refer to them collectively as nitrogen oxides (NO_x). While lumping the two nitrogen oxides together may be appropriate in some fields, such as atmospheric chemistry, it is inappropriate when looking at the biological activity of the molecules or their reactions in solution. The proposal that NO donors, such as S-nitroso-*N*-acetylpenicillamine (SNAP), sodium nitroprusside (SNP), and the host of others that have been developed in recent years, are really producing NO_x, as stated by Preston *et al.* (2004), would create no small amount of consternation in the medical and pharmaceutical arenas.

NO gas under ambient conditions does react rapidly ($k = 7 \times 10^3\,l^2\,M^{-2}\,s^{-1}$) with oxygen to form NO_2, which at $<100°C$ dimerizes reversibly to form dinitrogen tetroxide (N_2O_4) (Olbregts, 1985; Tsukahara *et al.*, 1999). While NO converts to NO_2 in air, in aqueous solution it is solely converted to nitrite (NO_2^-), and not $NO_2^- + NO_3^-$ as is commonly believed (Ignarro *et al.*, 1993) and stated by Preston *et al.* (2004). Under ambient conditions NO reacts rapidly ($k = 2 \times 10^6\,l^2\,M^{-2}\,s^{-1}$) to form NO_2^- in solution, according to the following stoichiometry:

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+$$

NO_2^- in solution reacts further to form nitrous acid (HNO_2), a weak and unstable acid. However, the amount of HNO_2 formed by nitrite in solution is variable and largely dependent on the pH of the solution. For instance, a study conducted by Braida and Ong (2000) found that at pH 2.85 67% of the nitrite present in solution was in the form of HNO_2, while at pH > 5.3 only 1% of the nitrite was present as HNO_2.

HNO_2, when formed, decomposes according to the stoichiometry:

$$HNO_2(aq) \leftrightarrow NO(g) + NO_2(g) + H_2O(l)$$

The products of HNO_2 decomposition can further react with water. NO, as described above, can react to form NO_2^- and HNO_2, dependent on the pH of the solution, or evolve out as NO gas, depending on the degree of aeration. However, NO_2 (or rather the dimerized form N_2O_4 at ambient conditions) reacts very differently in solution, forming equimolar amounts of NO_2^- and NO_3^- according to the stoichiometry:

$$N_2O_4(g) + H_2O(l) \leftrightarrow NO_2^- + NO_3^- + 4H^+$$

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NO_3^-, as the base of a strong acid, is disassociated across a wide range of pH with the conjugate, nitric acid (HNO_3) at the lowest pH. HNO_3 decays spontaneously to form NO_2. The amount of HNO_3 formed depends on the pH and degree of aeration of the system, and the consequent resident time of $NO_2(g)$ in the solution; decreased pH and increased aeration will lead to a net loss of nitrogen from the solution by the evolution of nitrogen oxide gases ($NO_x = NO + NO_2$) (Braida and Ong, 2000).

From the above chemistry it is clear that, rather than pH being a 'confounding factor' as asserted by Preston *et al.* (2004), it is a critical consideration in studies with nitrogen oxides and seed germination to understanding what species of nitrogen oxide is, or isn't, affecting germination.

NO donor

The NO-donor studies conducted by Preston *et al.* (2004) exposed seeds of both *Emmenanthe penduliflora* and *Nicotiana attenuata* to NO and the nitrite ion, with the possibility of some exposure to trace amounts of HNO_2 and even lesser amounts of the HNO_2 decomposition products, NO_2, NO_3^- and HNO_3. Exposure in the NO donor was over a period of 4 d and with an end concentration of $\leq 1\,\mu M - 2\,\mu M$ nitrite in three of their treatments, and a maximum of 42 μM in one treatment. These are likely reasonably true estimates of the concentrations, contrary to the authors' assertions that they are an underestimate, as they did not measure nitrate. It is doubtful, given the above chemistry, that any significant amount of nitrate formed in these solutions over the 4 d of testing.

The study had low germination in all NO-donor treatments, but it was significant with *N. attenuata*, in response to 100 μM SNAP. While these results are moderately interesting, they do not directly compare to any of the published studies by Keeley and Fotheringham (1997, 1998a, b, 2000). Despite their assertions otherwise, concentrations of NO employed by Preston *et al.* (2004) do not approach concentrations of similar treatments published by Keeley and Fotheringham (Table 1), nor are the experimental procedures comparable. For instance, the one published result by Keeley and Fotheringham (1997) utilizing NO was applied as a gas to dry seeds at 20.8 g m^{-3} (690 μM) for 30 s; because NO converts to NO_2 in air, calculations utilizing the above rate reaction yield the concentrations of NO and NO_2 to be 270 μM and 420 μM, respectively. Keeley and Fotheringham also conducted dry exposure of seeds to NO_2 gas alone at concentrations ranging from 17 μM to 167 μM for 0.5–1 min exposure times, followed by incubation in de-ionized water. In one of these treatments, 167 μM for 0.5 min, seeds were incubated in buffered solutions from pH 3 to pH 8. Whereas water incubation resulted in 100% germination, seeds incubated in buffer had the greatest germination at low pH, but this decreased to 20% at pH 7 and 0% at pH 8. Preston *et al.* (2004) suggest that longer exposures to lower doses are equivalent to shorter exposures to higher doses. There is no *a priori* reason to believe this is true and, in fact, the authors give good reason why this shouldn't be the case in their discussion. Post-fire specialists have evolved germination in response to fire-specific conditions; responding to lower levels of cues, which may occur at background levels between fires, would be selected against. Methodology and concentration differences between the NO studies render them incomparable, and the findings of Preston *et al.* (2004) cannot be used to refute earlier findings by Keeley and Fotheringham (1997).

Nitrite/nitrate

Nitrate and nitrite studies presented by Preston *et al.* (2004) are also not comparable to data presented by Keeley and Fotheringham (1997, 1998b). They conducted treatments with NO_3^- and NO_2^- equilibrated to pH 7 with an exposure time of 3 h. Germination in response to 100 μM NO_3^- or NO_2^-, when it occurred, was comparatively low in comparison to the smoke response. Keeley and Fotheringham (1998b) also conducted germination trials with NO_3^- and NO_2^-, but these were more extensive and were conducted across a range from pH 3 to pH 7, with exposure times from 3 h to continuous, and at a concentration of 1–10 mM. Germination was pH-dependent but, interestingly, highest at very low pH for NO_3^- (97%) and at *c.* pH 6 for NO_2^- (100%) (Table 1). At pH 7, Keeley and Fotheringham obtained $<10\%$ germination with any of these solutions.

Apparent exceptions to the lack of response in the Preston *et al.* (2004) study were the treatments referred to by the authors as 'extremely high', without any explanation as to why the authors considered this to be the case. These studies consist of an unknown number of treatments that were ≥ 10 mM NO_3^- and NO_2^-, presumably these were also for a 3 h exposure and equilibrated to pH 7, but this is not stated explicitly in the manuscript. There is no formal presentation of these data, and the authors' only comments were that they had germination comparable to smoke treatments. The fact that these data were not presented for reader review, when the methodology corresponds closest to studies conducted by Keeley and Fotheringham, and represent the only substantial positive germination results other than smoke, seems odd. A skeptic might be inclined to think that these results were glossed over because they did not conform to a predetermined goal of the study,

Table 1. Summary of experimental conditions and results by Preston *et al.* (2004) (upper) and Keeley and Fotheringham (1997, 1998a, b) (lower)

Summary of Preston *et al.* (2004) germination results regarding nitrogen oxides:

	Exposure time (h)	Final nitrogen oxide concentration	% Germination	
			N. attenuata	*E. penduliflora*
SNP				
10 μM	48	<1 μM NO_2^-	0	0
100 μM	48	<1 μM NO_2^-	~12	~4
SNAP				
10 μM	48	~2 μM NO_2^-	~8	0
100 μM	48	42 μM NO_2^-	~6	0
Nitrate ($NaNO_3$)	3	100 μM NO_3^-	~2	0
	unknown?	≥10 mM NO_3^-	~90?	N/A?
Nitrite ($NaNO_2$)	3	100 μM NO_2^-	~2	0
	unknown?	≥10 μM NO_2^-	~90?	N/A?

SNP, sodium nitroprusside; SNAP, S-nitroso-*N*-acetylpenicillamine; ? data not presented in manuscript.

Summary of Keeley and Fotheringham (1997, 1998a, b) germination results regarding nitrogen oxides (*E. penduliflora* only)

			% Germination							
				Buffered						
Solution	Concentration	Exposure duration	Unbuffered	pH	3	4	5	6	7	8
Nitrate (KNO_3)	1 mM	cont.	62		–	–	–	–	–	–
	10 mM	cont.	0		–	–	–	–	–	–
	10 mM	3 h	100		77	63	30	7	20	0
	10 mM	6 h	100		77	83	13	3	0	0
	10 mM	9 h	100		97	97	70	0	0	0
Nitrite ($NaNO_2$)	10 mM	cont.	–		0	0	0	0	0	–
	10 mM	cont.	–		0	0	0	20	10	–
	10 mM	6 h	–		0	0	0	100	0	–
	10 mM	12 h	–		0	0	60	90	0	–
Nitrite (KNO_2)	10 mM	cont.	–		0	0	0	0	0	–
Nitrogen dioxide (NO_2)	0.79 g m^{-3}	0.05 h	100		–	–	–	–	–	–
	7.7 g m^{-3}	0.008 h	100		44	64	28	20	0	–
	7.7 g m^{-3}	0.05 h	0		–	–	–	–	–	–
Nitrogen oxides (NO)	20.8 g m^{-3}	0.008 h	100		–	–	–	–	–	–
	690μM NO init. conc. = 270 μM NO + 420 μM NO_2 Final conc.)									

cont., Continuous; init., initial.

specifically to disprove any role for nitrogen oxides in the germination of *N. attenuata*.

Smoke

Preston *et al.* (2004) also conducted an apparently limited number of smoke treatments (again omitting any response curves that would allow one to distinguish insufficient from lethal levels), consisting of α-cellulose smoke and a 1:300 dilution of liquid smoke. These solutions were adjusted variously to pH 4, 5 and 7. The authors admit that germination was significantly greater in the liquid smoke at pH 4 than at pH 7, but assert that there was no significant difference between the cellulose smoke treatments at pH 5 and pH 7; the latter is not supported by the differences in the means and the size of the error bars presented in Fig. 1 of Preston *et al.* (2004).

While significant germination occurred in response to all of these treatments relative to water controls, none approached the 100% germination observed by Keeley and Fotheringham with 2 min direct smoke treatment, indirect transfer of smoke by smoke-treated filter paper or 1000:1 dilution of liquid smoke (unpublished data) with the same population of *E. penduliflora*.

Preston *et al.* (2004) assert that no nitrite was found in the smoke solutions; this seems anomalous as there is a very large body of literature indicating that both NO and NO_2 are significant components of plant biomass smoke. It is possible that smoked water samples were not tested for a significant period of time after production, and that much of the nitrite would have been lost to out-gassing. It is also possible that there was some other undetected error in their assay methodology. Employing the same assay methodology, studies by Keeley and Fotheringham found NO_3^-/NO_2^- levels in smoke-treated water to be 250–360 μM (not 167–375 μM as erroneously reported by Preston *et al.*, 2004). In addition, Keeley and Fotheringham found NO_3^-/NO_2^- levels in NO_2-treated water to be 350–375 μM [contrary to the assertions of Preston *et al.* (2004), Keeley and Fotheringham present no data on NO_3^-/NO_2^- levels for NO-treated water, as no such tests were ever conducted in our lab].

It is probable that the smoke from pure α-cellulose did not contain any nitrogen oxides, provided pyrolysis was at <2000°C, and there was no contamination. However, results from their nitrite assay are dubious and cannot be relied on to confirm this independently. The apparent motivation for using α-cellulose is a desire to disprove any role for nitrogen oxides in germination. This is really rather frivolous, as Keeley and Fotheringham (1997) never contended that nitrogen oxides were the only component of smoke to stimulate germination; in fact they also found other factors that occur in smoke, or can formed by dissolving smoke into solution, that are capable of stimulating germination, including CO_2 (putatively the primary product of combusting α-cellulose), as well as sulphuric acid (H_2SO_4), which forms when sulphur dioxide (SO_2) dissolves in water. In addition, Keeley and Fotheringham never indicated that this was an all-inclusive list of germination cues in smoke.

There are other procedural differences between the studies that make comparing them difficult. Many seeds are daylength and temperature-regime sensitive, and will not germinate, or have decreased germination, outside certain regimes. Germination conducted by Keeley and Fotheringham included 1 week of cold (5°C) stratification in dim light, followed by incubation for a 12L:12D 20°C/12°C day/night regime. This regime was chosen based on environmental conditions prevalent during the natural germination season of a winter annual such as *E. penduliflora.* Because seeds can vary in time to germination, seeds were assayed for ≥1 month, or until no further germination was observed. Preston *et al.* (2004) conducted germination under a regime of 16L:8D 27°C/23°C day/night with no cold stratification. Seeds were assayed for germination for 7–10 d. There is no reason given for this particular incubation regime, which would seem more appropriate for a summer annual species. To determine whether any of these factors cause significant critical differences would require further testing.

Clearly, while both Preston *et al.* (2004) and Keeley and Fotheringham (1997) tested germination in response to smoke and nitrogen oxides on *E. penduliflora*, the former studies are a very limited subset of the experiments conducted by the latter, primarily ones that had negative germination results in both studies. The findings of Preston *et al.* (2004), despite their assertions otherwise, cannot be used logically to refute any of the findings that nitrogen oxides play a role in smoke-stimulated germination in *E. penduliflora*, except under conditions as previously published (Keeley and Fotheringham, 1997, 1998a, b, 2000), specifically low response to nitrite and lack of response of nitrate at neutral pH.

With regard to *N. attenuata*, this species was never tested by Keeley and Fotheringham, and no speculation was made as to the stimulatory agent in smoke responsible for its germination. Certainly, all the publications by Keeley and Fotheringham indicated clearly that while certain nitrogen oxides induce germination in a limited number of California post-fire endemics, this did not apply to all the smoke-

stimulated species they tested. That said, the *N. attenuata* response to nitrites deserves more detailed study, including concentration and pH response curves.

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