A solution to the problem of ion confounding in experimental biology

To the editor: Understanding ion-specific effects is a central theme of biology. But because ions are generally manipulated through the use of salts, the complication of 'ion confounding' is introduced. Ion confounding occurs when the ion(s) of interest are covaried with the covaried ion associated with the salts used; that is, changing the concentration of a single cation or anion using a single salt results in a simultaneous change in the associated co-ion. If the concentration of an ion is not varied independently, then the main effect associated with that ion is indistinguishable from or confounded with, the effect(s) caused by changing the concentrations of the other ions. Identifying ion confounding requires converting reported salt formulations, including ions introduced via pH adjustments, to corresponding ion concentrations. If there are deviations of ion type and/or concentration other than those specified by the experimental design, then ion confounding has occurred. Ion confounding is extremely common in disciplines that study ion-specific effects, but is particularly apparent in plant nutrition studies, as the complex mineral-ion mixtures used are the primary nutritive source of these organisms (**Table 1**)[1].

To avoid ion confounding, it is necessary to be able to calculate a salt mix, or 'recipe', that achieves any given ionic formulation. This can be easily solved as a linear programming problem that optimizes the allocation of mixture components (the ions) from a set of salts. The linear function, presented as a minimization, can be used to determine the types and concentrations of salts, acids and bases to achieve a particular ionic solution:

$$\text{minimize} \qquad c_1X_1 + c_2X_2 + \ldots + c_nX_n \qquad (1)$$

where c is a known weighting coefficient, and X is the unknown amount of an individual salt,

$$\text{subject to} \quad a_{i1}X_1 + a_{i2}X_2 + \ldots + a_{in}X_n \{\leq, =, \geq\}\ b_i \quad (i = 1, 2, \ldots m)$$

$$X_1, \ldots X_n \geq 0$$

where a are known coefficients of ion proportionality for each salt X that contributes an ion defined by the target ion concentration b; a non-negative constraint ensures positive values. Equation (1) results in a list of the type and amount of salts to achieve the exact ion concentrations specified by b (**Supplementary Methods** online). A software application for making these calculations and generating associated pipetting tables is available from the authors.

Table 1 | Ion confounding of the hydroponic nutrient solutions

Solution	pH	NO_3^-	PO_4^{3-}	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}
Control	4.44	15	1	6	5	2	2.05
Lacking N	4.51	0[a]	1	**5**	**2.5**	2	**6.55**
Lacking PO_4^{3-}	4.54	**14**	0[a]	6	**4**	2	2.05
Lacking K^+	4.43	**10**	1	0[a]	**5.5**	2	2.05
Lacking Ca^{2+}	4.45	**5**	1	6	0[a]	2	2.05
Lacking Mg^{2+}	4.44	**14**	1	**10**	**4**	0[a]	**1.55**
Lacking SO_4^{2-}	4.27	**18**	1	**7**	**4**	2	0[a]

Ion confounding is illustrated by calculating the ion concentrations of the popular salt formulations developed by Hoagland and Arnon[1] for determining ion-specific effects on hydroponically grown plants. When the formulations are viewed in this manner the confounding becomes clear. For example, the formulation 'lacking Mg^{2+}' does not just change the $[Mg^{2+}]$ as would be required to determine the effect(s) of this ion, but compared to the control solution ion levels also changes the $[NO_3^-]$, $[K^+]$, $[Ca^{2+}]$ and $[SO_4^{2-}]$. Consequently, the measured responses cannot be attributed to Mg^{2+} alone, but will be a product of the combined changes of multiple ions; the Mg^{2+} main effect is now confounded with the effect(s) of the covaried ions. Only major mineral ions are shown; Cl^-, Fe^{2+}, Zn^{2+} and Cu^{2+} are also confounded. pH calculated using MINEQL+ ver 4.5 (Environmental Research Software, Hallowell, Maine, USA) chemical equilibrium modeling system. Ion levels are in mM. Confounded ions are in bold.

[a]Ions being varied.

Equation (1) provides the means to generate exact solutions of complex ion mixtures. Thus, ions can and should be manipulated as independent factors in statistically valid experiments (**Supplementary Note** online). The overarching impact of ion confounding is that our understanding of ion-specific effects on biological processes is primarily based on flawed experimental designs. This implies that the most basic ion-specific effects on biological responses have never been properly characterized (**Supplementary Discussion** online). The approach presented here provides the means to overcome this fundamental hindrance to valid experimental study of ion-specific effects.

Note: Supplementary information is available on the Nature Methods website.

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1. Hoagland, D.R. & Arnon, D.I. The water-culture method for growing plants without soil. *Univ. Calif. Coll. Agric. Exp. Stn. Circ.* **347**,1–39 (1938).

Supplementary Methods. Solving Equation (1) – An Example.

To illustrate the mechanics of using equation (1), a simple ionic solution containing 40 mM NO_3^- , 5 mM NH_4^+ , 1 mM PO_4^{3-} , 2 mM Cl^-, and 20 mM K^+ is formulated from a selection of six salts - KNO_3, NH_4NO_3, $(NH_4)_2 \cdot HPO_4$, $Ca(NO_3)_2 \cdot 4H_2O$, KCl, and KH_2PO_4. More salts may be added to the equation, and will serve to increase the number of possible salt combinations that satisfy the defined ionic solution. A software program developed by the authors, ARS-MEDIA, is available to make these calculations using equation (1). ARS-MEDIA will also generate a pipetting table from the resulting solution. All the parameters in equation (1) can be varied in ARS-MEDIA thereby allowing formulations that are convenient to the user such as availability, cost, or solubility.

Step 1	Construct the linear objective function using the desired salts. The objective function for this problem is as follows: **Minimize:** mM KNO_3 + mM NH_4NO_3 + mM $(NH_4)_2 \cdot HPO_4$ + mM $Ca(NO_3)_2 \cdot 4H_2O$ + mM KCl + mM KH_2PO_4 The function defines the fewest number of salts that will result in the target ionic solution as defined in the constraint component of the equation.
Step 2	Since the types and concentrations of salts required are unknown, replace each salt with a variable (i.e., $X_1 + X_2 + \ldots + X_n$). The equation is as follows: **Minimize:** $X_1 + X_2 + X_3 + X_4 + X_5 + X_6$
Step 3	The default weighting is one. However, weighting the individual salts by adding a coefficient other then one to some or all of the salt variables will result in a medium recipe that may be of more practical value. For example, to determine the cheapest combination of salts, the cost (e.g., price/gram) would be added to each salt variable as follows:

Minimize: $0.0655X_1 + 0.0638X_2 + 0.214X_3 + 0.0858X_4 + 0.046X_5 + 0.093X_6$

The above coefficients represent US$/gram from Sigma-Aldrich Co. On a practical note, solving for the cheapest combination of salts is a simple method to reduce operational costs for commercial applications that use large quantities of salts.

Step 4 Construct the constraints on the objective linear function. Individual constraints are defined as the desired ion concentrations (the right-hand side of the equations below). The constraint coefficients, ***a***, are derived from the Constraint Coefficient Table illustrated below. Note that these coefficients represent the proportion of the target ion contributed by each salt. A non-negativity constraint is also defined for each salt to prevent equation (1) from generating negative solutions. Each constraint is constructed from its respective column in the coefficient table.

$9.8909X_1 + 12.4933X_2 + 0X_3 + 8.4692X_4 + 0X_5 + 0X_6 = 40$ mM $\mathbf{NO_3^-}$

$0X_1 + 12.4933X_2 + 15.1434X_3 + 0X_4 + 0X_5 + 0X_6 = 5$ mM $\mathbf{NH_4^+}$

$0X_1 + 0X_2 + 7.5717X_3 + 0X_4 + 0X_5 + 7.3483X_6 = 1$ mM $\mathbf{PO_4^{3-}}$

$0X_1 + 0X_2 + 0X_3 + 0X_4 + 13.4136X_5 + 0X_6 = 2$ mM $\mathbf{Cl^-}$

$9.8909X_1 + 0X_2 + 0X_3 + 0X_4 + 13.4136X_5 + 7.3483X_6 = 20$ mM $\mathbf{K^+}$

$X_1 \geq 0$

$X_2 \geq 0$

$X_3 \geq 0$

$X_4 \geq 0$

$X_5 \geq 0$

$X_6 \geq 0$

Step 5	The completed equation is as follows and can be solved by any linear programming application. It should be noted that some spreadsheets such as Microsoft Excel include a linear programming solving function.

Minimize: $X_1 + X_2 + X_3 + X_4 + X_5 + X_6$

Subject to:

$9.8909X_1 + 12.4933X_2 + 0X_3 + 8.4692X_4 + 0X_5 + 0X_6$ = 40 mM **NO_3^-**

$0X_1 + 12.4933X_2 + 15.1434X_3 + 0X_4 + 0X_5 + 0X_6$ = 5 mM **NH_4^+**

$0X_1 + 0X_2 + 7.5717X_3 + 0X_4 + 0X_5 + 7.3483X_6$ = 1 mM **PO_4^{3-}**

$0X_1 + 0X_2 + 0X_3 + 0X_4 + 13.4136X_5 + 0X_6$ = 2 mM **Cl^-**

$9.8909X_1 + 0X_2 + 0X_3 + 0X_4 + 13.4136X_5 + 7.3483X_6$ = 20 mM **K^+**

$X_1 > 0$

$X_2 > 0$

$X_3 > 0$

$X_4 > 0$

$X_5 > 0$

$X_6 > 0$

Step 6	The resulting recipe specifies how many grams/liter of each salt is required to make the target ionic solution:

X_1: KNO_3 = 1.718752

X_2 : NH_4NO_3 = 0.400214

X_3 : $(NH_4)_2 \cdot HPO_4$ = 0.000000

X_4 : $Ca(NO_3)_2 \cdot 4H_2O$ = 2.125348

X_5 : KCl = 0.149102

X_6 : KH_2PO_4 = 0.136086

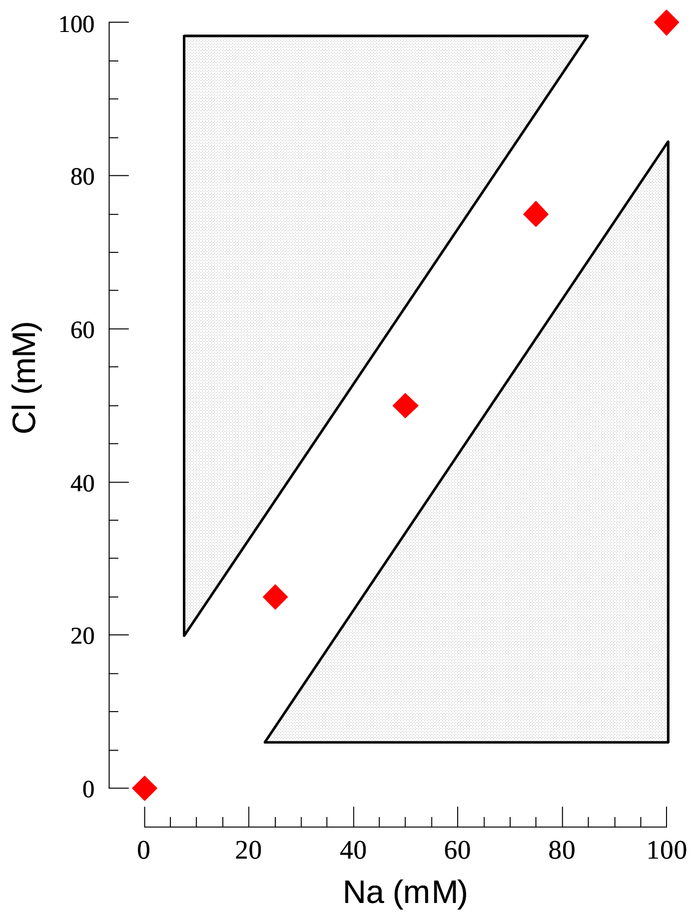
Constraint Coefficient Table					
Objective function variables (i.e., the salts)	mM[1] (1 g)	Ions (mM)[2]			
		NO_3^-	NH_4^+	PO_4^{3-}	K^+
KNO_3 (X_1)	9.8909	9.8909	0	0	9.8909
NH_4NO_3 (X_2)	12.4933	12.4933	12.4933	0	0
$(NH_4)_2{\cdot}HPO_4$ (X_3)	7.5717	0	15.1434	7.5717	0
$Ca(NO_3)_2{\cdot}4H_2O$ (X_4)	4.2346	8.4692	0	0	0
KCl (X_5)	13.4136	0	0	0	13.4136
KH_2PO_4 (X_6)	7.3483	0	0	7.3483	7.3483

[1] The mM value of 1 gram of the corresponding salt.
[2] The mM value of each anion and cation from 1 gram of the corresponding salt.

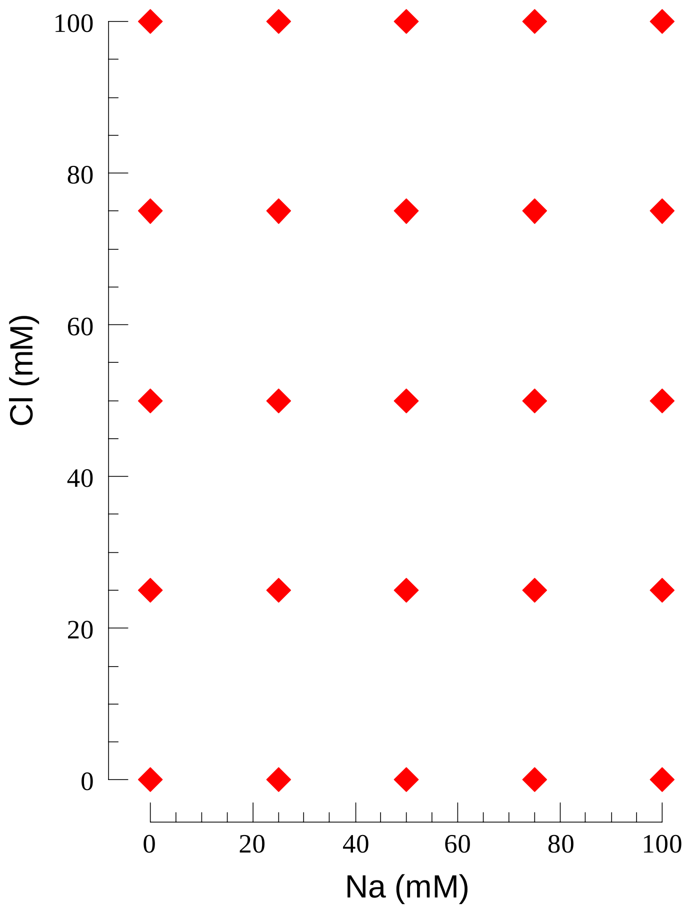
The ion concentrations are derived from the ion equivalents for each salt. For example, the salt $(NH_4)_2HPO_4$ has two equivalents of NH_4 and one equivalent of PO_4; therefore, the mM level is calculated by multiplying the ion's equivalent times the mM value of 1 gram of the salt. For $(NH_4)_2HPO_4$ the calculation is as follows: 16 mM $(NH_4)_2$ = 2 equivalents x 8 mM/g $(NH_4)_2HPO_4$, and 8 mM PO_4 = 1 equivalent x 8 mM/g $(NH_4)_2HPO_4$. Therefore, 1 gram of the salt $(NH_4)_2HPO_4$ contributes 16 mM of NH_4 and 8 mM of PO_4 when added to a solution.

Supplementary Note. Salt- versus ion-based experimental design space.

Consider the design of a simple experiment to determine the ion-specific effects of Na^{+} and Cl^{-} on a particular biological response. A common salt-based approach will vary the concentration of the salt NaCl followed by measurement of various responses such as growth and tissue levels of Na^{+} and Cl^{-}. By using the salt NaCl as the independent variable (0, 25, 50, 75, and 100 mM) rather than the ions Na^{+} and Cl^{-}, the possible experimental design space is inadequately sampled as figured below. Design points occur along the center diagonal of the 2-factor design space; the majority of the possible design space, shown above and below the center diagonal as the shaded regions, remains unexplored. Ion-specific main effects and interactions cannot be determined. As additional ions are added to the study the problem of unexplored design space is extended into those dimensions.



Conversely, an experimental design where the ions are the independent factors and equation (1) is used to generate the formulations necessary for all the design points results in an adequate sampling of the design space. The main effects of Na^+ and Cl^- and the interaction effect of Na^+ and Cl^- can now be statistically determined for each measured response.



Supplementary Discussion. Representative examples of ion confounding.

Ion confounding is extremely common in all disciplines concerned with ion-based research and are too numerous to comprehensively cover here. However, the following examples are representative of the biological literature. It is worth noting that unlike many studies, these examples generally provide sufficient information to make the necessary ion comparisons for analysis. Another common type of ion confounded research is termed "co-ion substitution." This is a method wherein multiple salts with a common ion (e.g. Cl^-) but with varying counterions (e.g. K^+, Ca^{2+}, Na^+, Li^+) are used to discern single ion effects from salt-based manipulations (see 2, 5, 11, 13, 16, and 21 below). This approach requires the assumption that all interactions between the common ion and all counterions are equivalent; as far as we know, this assumption has never been experimentally verified for any biological response. There are also studies that technically exhibit ion confounding, but may not be as egregious as the previous examples; for instance, when the counterion(s) is present at significantly higher concentration(s) relative to the ion(s) of interest, e.g. manipulating any ions (NO_3^-, PO_4^{3-}, or trace metals) at μM levels in seawater with Na^+ and/or Cl^- salts.

1. Kingsbury, R.W. & Epstein, E. Salt sensitivity in wheat – a case for specific ion toxicity. *Plant Physiol.* **80**, 651-654 (1986).

   The objective was "to determine which of the two ions most frequently implicated in salinity, Na^+ and Cl^-, is most toxic to wheat." Three tables are presented, the first reproduces information as reported (confounded ions are in boldface type), the second illustrates what these solutions would look like with ion confounding removed, and the third table shows salt formulations generated from equation (1) that result in the ionic solutions specified in the second table. All ions confounded except Na^+ in Hoagland-Cl.

   As reported – with ion confounding – all ions confounded except Na^+ in Hoagland-Cl (i.e., compared to Hoagland control).

	Hoagland mM	Na-Hoagland mM	Hoagland-Cl mM	NaCl mM
NO_3^-	182	**121**	**14**	**14**
SO_4^{2-}	25	**15**	**1**	**1**
PO_4^{3-}	14	**9**	**2**	**2**
Cl^-	0.05	**0.05**	**211**	**125**
NH_4^+	14	**2**	**11**	**2**
K^+	78	**6**	**60**	**6**
Ca^{2+}	52	**4**	**40**	**4**
Mg^{2+}	25	**1**	**39**	**1**
Na^+	0	142	0	**125**

Ion confounding removed. The only difference between the four solutions is the ions of interest, Na^+ and Cl^-.

	Hoagland mM	Na-Hoagland mM	Hoagland-Cl mM	NaCl mM
NO_3^-	182	182	182	182
SO_4^{2-}	25	25	25	25
PO_4^{3-}	14	14	14	14
Cl^-	0.05	0	142	71
NH_4^+	14	14	14	14
K^+	78	78	78	78
Ca^{2+}	52	52	52	52
Mg^{2+}	25	25	25	25
Na^+	0	142	0	71

One set of nonunique salt formulations derived from equation (1) results in the ionic solutions listed above and removes ion confounding. Approximately 30 salts, acids, and bases were used to solve equation (1).

Salts	Hoagland mg L^{-1}	Na-Hoagland mg L^{-1}	Hoagland-Cl mg L^{-1}	NaCl mg L^{-1}
$CaCl_2 \cdot 2H_2O$				5,219.56
$Ca(NO_3)_2.4H_2O$	12,279.85	12,279.85	12,279.85	3,896.49
H_2SO_4	2.45			
HCl			5,177.32	
K_3PO_4		2,971.77		
KH_2PO_4	1,905.20		1,905.20	1,905.20
K_2HPO_4				0.003
KNO_3	6,470.59	3,639.71	6,470.59	6,470.59
$MgCl_2.6H_2O$	5.08			
$MgSO_4.7H_2O$	6,155.56	6,161.73	6,161.73	6,161.73
$NaNO_3$		3,569.61		6,034.34
NaOH		3,999.70		
NH_4NO_3	1,120.60		1,120.60	1,120.60
NH_4OH		490.70		

2. Hoagland, D.R. & Arnon, D.I. The water-culture method for growing plants without soil. *Univ. Calif. Coll. Agric. Exp. Stn. Circ.* 347 (1938).

Explained in Table 1 of manuscript.

3. Liu, J. & Zhu, J-K. An *Arabidopsis* mutant that requires increased calcium for potassium nutrition and salt tolerance. *Proc. Natl. Acad. Sci. USA* **94**, 14960-14964 (1997).

The *Arabidopsis sos3* mutant was isolated and its Ca^{2+}, K^+, Na^+, and Cl^- requirements characterized.

To determine the *sos3* mutant K^+ requirement, seedlings were grown on potassium-free 1/20th MS salts supplemented with varying amounts of KCl. To remove the potassium required omitting KNO_3 and KH_2PO_4 and adding $(NH_4)_2HPO_4$ to restore the PO_4^{3-}. Compared to 1/20th MS salts without ion confounding the net result was that the effect of removing K^+ was confounded with the reduction in NO_3^- and increase in NH_4^+. Additionally, Cl^- was not held constant but varied along with the K^+ due to the varying amounts of KCl used.

To determine the *sos3* mutant Ca^{2+} requirement, seedlings were grown on calcium free 1/20th MS salts containing only 20 μM K^+ and supplemented with varying amounts of $CaCl_2$. One objective was to determine seedling growth under very low K^+ levels. The final solution was similar to the one above with the removal of KNO_3 and KH_2PO_4 and the adding of $(NH_4)_2HPO_4$ to restore the PO_4^{3-}. Compared to 1/20th MS salts without ion confounding the net result was that the effect of removing Ca^{2+} and lowering K^+ was confounded with a reduction in NO_3^- and an increase in NH_4^+. Additionally, Cl^- was not held constant but varied along with the Ca^{2+} due to the varying amounts of $CaCl_2$ used.

4. Tarakcioglu, C. & Inal, A. Changes induced by salinity, demarcating specific ion ratio (Na/Cl) and osmolality in ion and praline accumulation, nitrate reductase activity, and growth performance of lettuce. *J. Plant Nutrition* **25**, 27-41 (2002).

   The objective was "to separate the osmolalitic effect from the specific ion effects of Na^+, Cl^-, and Na/Cl ratio…" Two tables are presented, the first reproduces information reported and the second illustrates what these solutions would look like with ion confounding removed.

   As reported - with ion confounding (compare to control ion levels).

	NO_3^-	SO_4^{2-}	PO_4^{3-}	Cl^-	NH_4^+	K^+	Ca^{2+}	Mg^{2+}	Na^+
Control	19	1.125	2	0	1.25	11	4.5	1	0
NaCl (40 mM)	19	1.125	2	40	1.25	11	4.5	1	40
Na/Cl (100/0)	19	**21.125**	2	0	1.25	11	4.5	1	40
Na/Cl (75/25)	19	**16.125**	2	10	1.25	**17**	**5.5**	**2**	30
Na/Cl (50/50)	19	**11.125**	2	20	1.25	**21**	**7.5**	**3**	20
Na/Cl (25/75)	19	**6.125**	2	30	1.25	**31**	**7.5**	**3**	10
Na/Cl (0/100)	19	1.125	2	40	1.25	**32**	**12**	**3**	0

   Ion confounding removed.

	NO_3^-	SO_4^{2-}	PO_4^{3-}	Cl^-	NH_4^+	K^+	Ca^{2+}	Mg^{2+}	Na^+
Control	19	1.125	2	0	1.25	11	4.5	1	0
NaCl - (40 mM)	19	1.125	2	40	1.25	11	4.5	1	40
Na/Cl (100/0)	19	1.125	2	0	1.25	11	4.5	1	40
Na/Cl (75/25)	19	1.125	2	10	1.25	11	4.5	1	30
Na/Cl (50/50)	19	1.125	2	20	1.25	11	4.5	1	20
Na/Cl (25/75)	19	1.125	2	30	1.25	11	4.5	1	10
Na/Cl (0/100)	19	1.125	2	40	1.25	11	4.5	1	0

5. Takahashi, H. *et al.* Regulation of sulfur assimilation in higher plants: A sulfate transporter induced in sulfate-starved roots plays a central role in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. USA* **94**, 11102-11107 (1997).

The objective was to induce a sulfate transporter in sulfate-starved roots of *Arabidopsis*. The solution used to induce SO_4^{2-} starvation was formulated by replacing the sulphate salts with "equivalent amounts of chloride salts." This removes SO_4^{2-} but also increases the [Cl^-] from 5.9 mM to 9.6 mM. The measured response is confounded with the changed [SO_4^{2-}] and [Cl^-].

6. Ellison, A.M. & Gotelli, N.J. Nitrogen availability alters the expression of carnivory in the northern pitcher plant, *Sarracenia purpurea*. *Proc. Natl. Acad. Sci. USA* **99**, 4409-4412 (2002).

One objective was to determine the "direct effects of N additions on morphology of *S. purpurea*." Nine nutrient formulations were used. Treatments number 3 and 4 increased both NH_4^+ and Cl^- thereby confounding the individual effects of NH_4^+ and Cl^-. Treatments 5 and 6 increased both Na^+ and PO_4^{3-} thereby confounding the individual effects of Na^+ and PO_4^{3-}. Treatments 7, 8, and 9 varied the N/P ratio but no information is provided on how this was done.

7. Ruiz, D., Martinez, V. & Cerdá. Demarcating specific ion (NaCl, Cl^-, Na^+) and osmotic effects in the response of two citrus rootstocks to salinity. *Sci. Hort.* **80**, 213-224 (1999).

Documented directly in Table 1 of the paper. The objective was "separate the osmotic effect from specific ion effects of Cl^- and Na^+." The ions NO_3^-, SO_4^{2-}, K^+, Ca^{2+}, and Mg^{2+} were altered in addition to the concentration of NaCl, Cl^-, and Na^+.

8. Schulze, E.-D. & Bloom, A.J. Relationship between mineral nitrogen influx and transpiration in radish and tomato. *Plant Physiol.* **76**, 827-828 (1984).

Objective was to determine various responses of radish and tomato to the form of mineral nitrogen used. For radish there were two treatment nutrient solutions. The first contained 20 μM each of NO_3^-, NH_4^+, Cl^-, and K^+. The second contained 20 μM each of NH_4^+ and Cl^- and 200 μM each of NO_3^- and K^+. In addition to increasing the [NO_3^-], the [K^+] was also increased. Therefore, any effects associated with NO_3^- are confounded with the concomitant increase in K^+.

For tomato there were two treatment nutrient solutions. The first contained 200 μM NO_3^-, NH_4^+, Cl^-, and K^+. The second contained 1000 μM NO_3^-, NH_4^+, Cl^-, and K^+. In addition to increasing the [NO_3^-] and [NH_4^+], the [Cl^-] and [K^+] were also altered. Therefore, any effects associated with increased N are confounded with the concomitant increase in Cl^- and K^+. If nitrogen form was of interest then there is additional confounding introduced by not keeping the N forms separate.

9. Sasseville, D.N. & Mills, H.A. N form and concentration: effects on N absorption, growth, and total N accumulation with southern peas. *J. Amer. Soc. Hort. Sci.* **104**, 585-591 (1979).

The objective was to determine the effects of N form and amount on various responses of southernpeas. Total N was either 75 or 150 ppm and within each concentration the NO_3^-: NH_4^+ ratio was varied from 100:0 to 0:100. Two salts were used to construct these solutions – $Ca(NO_3)$ and $(NH_4)_2SO_4$. The result is that Ca^{2+} and SO_4^{2-} were simultaneously varied. Therefore, any effects associated with amount and proportions of N were confounded with the effects of varying Ca^{2+} and SO_4^{2-}.

10. Melo-Oliveira, R. *et al.* Arabidopsis mutant analysis and gene regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. *Proc. Natl. Acad. Sci. USA* **93**, 4718-4723 (1996).

One experiment measured GDH expression of a GDH deficient mutant at 0, 20, or 40 mM NH_4^+. NH_4^+ levels were varied using NH_4NO_3 thereby confounding the effect of NH_4^+ with the concomitant changes in the $[NO_3^-]$.

11. Zhu, J.-K. *et al.* Genetic analysis of salt tolerance in Arabidopsis: evidence for a critical role of potassium nutrition. *The Plant Cell* 10, 1181-1191 (1998).

Four cations were examined, Na^+, K^+, Li^+, and Cs^+ and their effects determined on salt tolerance. Co-ion substitution using the chloride salts of these ions was used. Therefore, the observed effects include cation-specific effects confounded with the interaction with the anion Cl^-.

12. Cramer, G.R., Epstein, E., Läuchli, A. Effects of sodium, potassium and calcium on salt-stressed barley. I. Growth analysis. *Physiol. Plant.* **80**, 83-88 (1990).

The objective was to assess the effects of "supplemental Ca^{2+} on longer-term growth (1 month)…" Six solutions were prepared and four ions varied – K^+, Ca^{2+}, Na^+, and Cl^- ; because of ion confounding it is not clear what can be determined from these combinations. The minimum number of treatment solutions (i.e., sufficient to estimate linear effects) would require at least 10 treatments, and 20 to estimate a quadratic model.

	K^+	Ca^{2+}	Na^+	Cl^-
A – 1/10 Hoaglands	0.6	0.4	0.005	0
B – A + 10 mM $CaCl_2$	0.6	10	0.005	20
C – A + 125 mM KCl	125	0.4	0.005	125
D – A + 125 mM NaCl	0.6	0.4	125	125
E – A + 125 mM KCl + 10 mM $CaCl_2$	125	10	0.005	145
F – 125 mM NaCl + 10 mM $CaCl_2$	0.6	10	125	145

13. Lo Nostro, P. *et al.* Specific ion effects on the growth rates of *Staphylococcus aureus* and *Pseudomonas aeruginosa. Phys. Biol.* 2, 1-7 (2005).

Twenty-one anions were examined and their effects determined on growth rates of two bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Co-ion substitution using the sodium salts of these ions was used. Therefore, the observed effects include anion-specific effects confounded with the interaction with the cation Na^+.

14. Elkonin, L.A. & Pakhomova, N.V. Influence of nitrogen and phosphorus on induction embryogenic callus of sorghum. *Plant Cell Tiss. Org. Cult.* **61**, 115-123 (2000).

The objective was to "study the regeneration ability of embryogenic cultures grown on modified media with increased levels of NO_3^-, NH_4^+, and PO_4^{3-}." However, K^+ and SO_4^{2-} levels were also changed thereby confounding the effects associated with the changes in in NO_3^-, NH_4^+, and PO_4^{3-}. It is worth mentioning that even without ion confounding the experimental design is not suitable for determining the effects of NO_3^-, NH_4^+, and PO_4^{3-} as the power of the design is less than 10% for detecting even a two standard deviation difference.

	K^+	NO_3^-	NH_4^+	PO_4^{3-}	SO_4^{2-}	Total N
MS	20.55	39.9	20.6	1.25	1.5	60.5
M2	**11.15**	72.4	62.5	1.25	1.5	134.9
M21	**53.15**	72.5	20.6	1.25	1.5	93.1
M23	**8.75**	39.9	47.6	1.25	**17.6**	87.5
M24	**7.85**	131.6	125	1.25	1.5	256.6
M11	**28.12**	39.9	20.6	8.82	1.5	60.5
M12	**70.62**	82.4	20.6	8.82	1.5	103
M14	**63.05**	82.4	20.6	1.25	1.5	103
M17	**47.72**	80.1	41.2	8.82	1.5	121.3
M18	**40.9**	80.1	41.2	2	1.5	121.3
M19	**42.84**	39.9	3.5	2.94	**4.25**	43.4
M20	**65.94**	63	3.5	2.94	**4.25**	66.5
N6	**30.94**	28	3.5	2.94	**4.25**	31.5

15. Appenroth, K.-J. & Gabrys, H. Ion antagonism between calcium and magnesium in phytochrome-mediated degradation of storage starch in *Spirodela polyrhiza*. *Plant Sci.* **165**, 1261-1265 (2003).

The objective was to determine the role of Ca^{2+} and the competition between Ca^{2+} and Mg^{2+} in phytochrome-mediated storage starch degradation. The only information on the treatments is, "$Ca(NO_3)_2$ and $MgSO_4$ were replaced by $NaNO_3$ and Na_2SO_4, respectively." Hence, precise ion composition tables cannot be constructed. However, it is clear that, at a minimum, in addition to changing [Ca^{2+}] and [Mg^{2+}] the [Na^+] was also altered.

16. Ohta, D. *et al.* Early responses of sodium-deficient *Amaranthus tricolor* L. plants to sodium application. *Plant Physiol.* **84**, 112-117 (1987).

The objective was to determine the "early responses of sodium-deficient *Amaranthus tricolor* plants to sodium application …" The effect of Na^+ is confounded both with the effect of increasing Cl^- and the Na^+ x Cl^- interaction. This study is an example of co-ion substitution as they also used KCl to vary K^+; Na^+ specific effects were inferred from the differential response.

17. Stewart, A.J. *et al.* The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. *Plant, Cell Environ.* **24**, 1189-1197 (2001).

The objective was to determine if flavonol accumulation would be affected by altering the nitrogen or phosphorus nutrition of *Arabidopsis* and tomato. A number of studies were conducted but only ion confounding associated with the experiment where the nitrogen content of MS media was altered will be explained.

Total N was varied from 0 to 60 mM (MS is 60 mM) and the ratio of NH_4^+ to NO_3^- was maintained at 1:2, the same as MS medium. The only salts used to vary the ratio were NH_4NO_3 and KNO_3. The result would be that K^+ levels were also changed along with the NH_4^+ to NO_3^-. For example, at 0 mM total N there would only be 1.25 mM K^+ versus 20 mM in standard MS – a reduction > 90%. There is some indication that K^+ may have been adjusted back to 20 mM with KCl, though this is not clear in the paper. If true, than the confounding shifts to a large increase in Cl^-.

18. Kang, J. & Turano, F.J. The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **100**, 6872-6877 (2003).

In one experiment the wild-type phenotype of antiAtGLR1.1 transgenic plants was restored by the addition of 5 mM NH_4NO_3 and 5 mM KNO_3. The authors concluded that the restored phenotype was due to the addition of N; however, potassium levels were confounded with the effects of the increased N via the use of KNO_3.

19. Martínez-Cordero, M.A., Martínez, V., Rubio, F. High-affinity K^+ uptake in pepper plants. *J. Exp. Bot.* **56**, 1553-1562 (2005).

K^+ depletion was examined with two experiments. In the first experiment pepper plants were transferred to a nutrient solution deprived of K^+. Compared to the control, K^+ was reduced from 1.4 mM to 0 mM, but the effect was confounded via a concomitant increase in Ca^{2+} from 0.8 to 1.5 mM.

In the second experiment pepper plants were transferred to a nutrient solution deprived of K^+ and supplemented with NH_4^+. Compared to the control, K^+ was reduced to 0 mM and 1.4 mM NH_4^+ added, but the effect was confounded via a concomitant decrease in NO_3^- from 2.8 to 1.4 mM and the addition of 1.4 mM Cl^-.

20. Leljak-Levanić, D., Bauer, N., Mihaljević, S., Jelaska, S. Somatic embryogenesis in pumpkin (*Cucurbita pepo* L.): Control of somatic embryo development by nitrogen compounds. *J. Plant Physiol.* **161**, 229-236 (2004).

The objective was to determine the effects of NH_4^+ and NO_3^- on somatic embryo development of pumpkin embryogenic callus. In addition to changing the [NH_4^+] and [NO_3^-], the [Cl^-], [K^+], and [Na^+] were also changed. This does not include the additional confounding introduced from ion changes via the unaccounted for pH adjustments.

	NH_4^+	NO_3^-	Cl^-	K^+	Na^+
MS	20	40	6	20	0.2
1 mM NH_4Cl	1	0	**7**	**1.25**	0.2
1 mM NH_4Cl + 55 mM KNO_3	1	55	**7**	**55**	0.2
60 mM KNO_3	0	60	6	**60**	0.2
60 mM $NaNO_3$	0	60	6	**1.25**	**60**

21. Lo Nostro, P. et al. Hofmeister specific ion effects in two biological systems. *Curr. Opin. Colloid Interf. Sci.* **9**, 97-101 (2004).

Fifteen anions were examined and their effects determined on wool water absorbency and growth rates of *Staphylococcus aureus.* Co-ion substitution using the sodium salts of these ions was used. Therefore, the observed effects include anion-specific effects confounded with the anion x Na^+ interaction. This is the only paper we found where the authors specifically mentioned the co-ion substitution assumption that the anion x Na^+ interaction effect is equal for both anions.