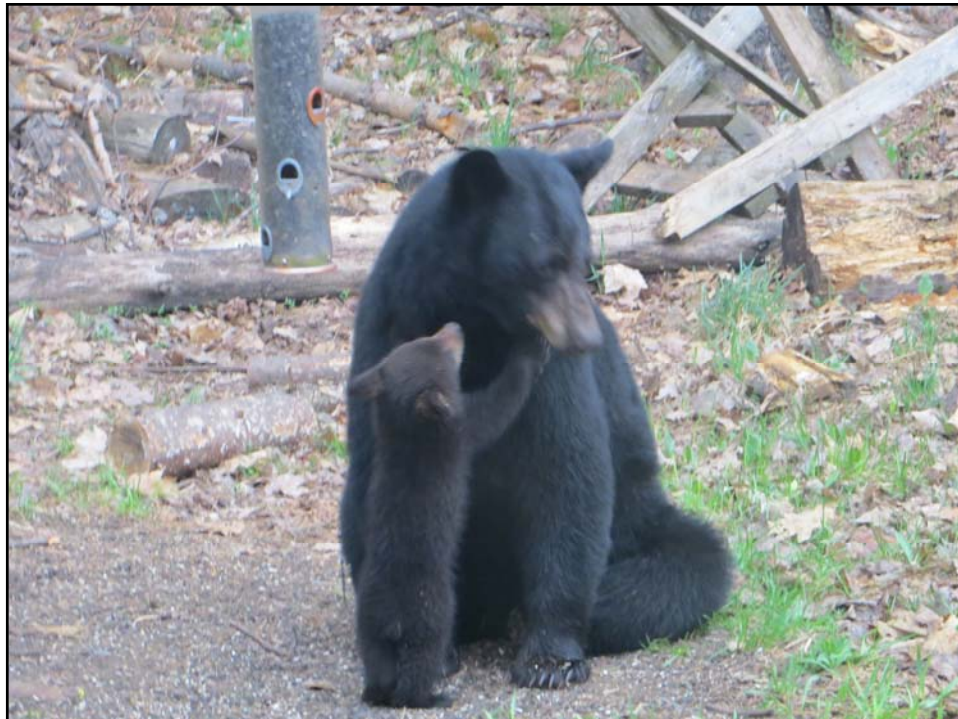


The Nitrate Elimination Co., Inc.



Analytical Grade Enzymes:
Biotechnology *Chemists* can Use





NECi is an environmental biotechnology company.

**We are dedicated to the application
of biotechnology to the solution of
environmental problems.**

**NECi's niche: Reagent Grade Enzymes
for Green Analytical Chemistry.**



NECi's History

Founded in 1993, based on:

- System for removal of nitrate from drinking water (*Nature 1992*).
- Dr WH Campbell's academic research on nitrogen metabolism in crop plants.
- Founders' expertise in recombinant protein expression.



NECi Base Technology – *and what we DO with it*

- Expression of complex recombinant proteins
- Subset = design of super-efficient protein catalysts, aka *Enzymes*
- Specialty = Enzymes for Analytical Chemistry
 - Is my food safe?
 - Are my drugs pure?
 - Is my water clean?
 - Is my process working?
- We develop and produce modern tools for today's analytical chemists
- We *simplify* these products for use outside the lab

NECi's R&D Funding

Small Business Innovation Research Program

- NIH funded development of recombinant nitrate reductase (AtNaR and YNaR)
- USDA funded development of field kits for testing nitrate toxicity in livestock feed
- USDA funded validation of enzyme-based nitrate detection reagent kits for Discrete Analyzers (lab robots), a collaboration with the USGS
- USDA funded enzyme-based test kits for phosphate in soil and water
- NSF funded hand held, smartphone-based photometer to pair with N and P test kits



Analytical Advantages of Enzymes

- **Selectivity**
"Find" target in complex mixtures
- **Sensitivity**
Low detection limits in complex mixtures
- **Specificity**
False negatives *and* false positives are rare
- **Safety**
For shipping, storage, handling, and disposal.



Reagent Grade Enzymes are accurate, reliable, and environmentally benign.

NECi Production of Nitrate Reductase by Fermentation



Recombinant Nitrate Reductase

- **Increased production capacity**
- **Guaranteed lot-to-lot reproducibility**
- **Reasonable production costs**
- **Engineered for improved stability**
 - **Allows storage at controlled RT**
 - **Stable during analytical runs**
 - **Shipping at ambient**



How the Enzyme-based Nitrate Assay Works

Nitrate reductase (**NaR**) catalyzes the reduction of nitrate to nitrite. The B vitamin **NADH** is cofactor.

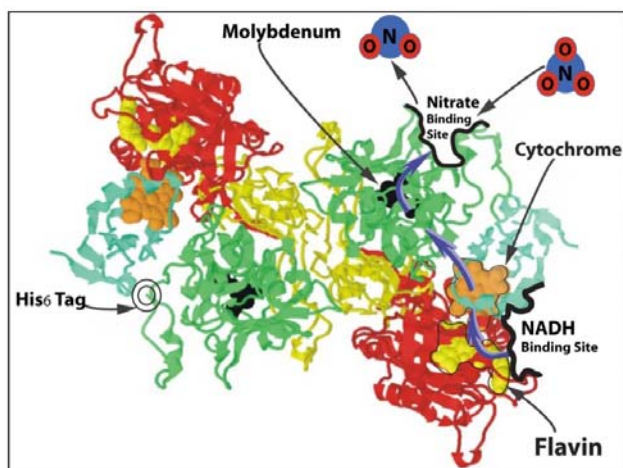
The resulting **nitrite** reacts with the Griess color reagents to produce a highly colored **product**. Absorbance measured at 540nm.

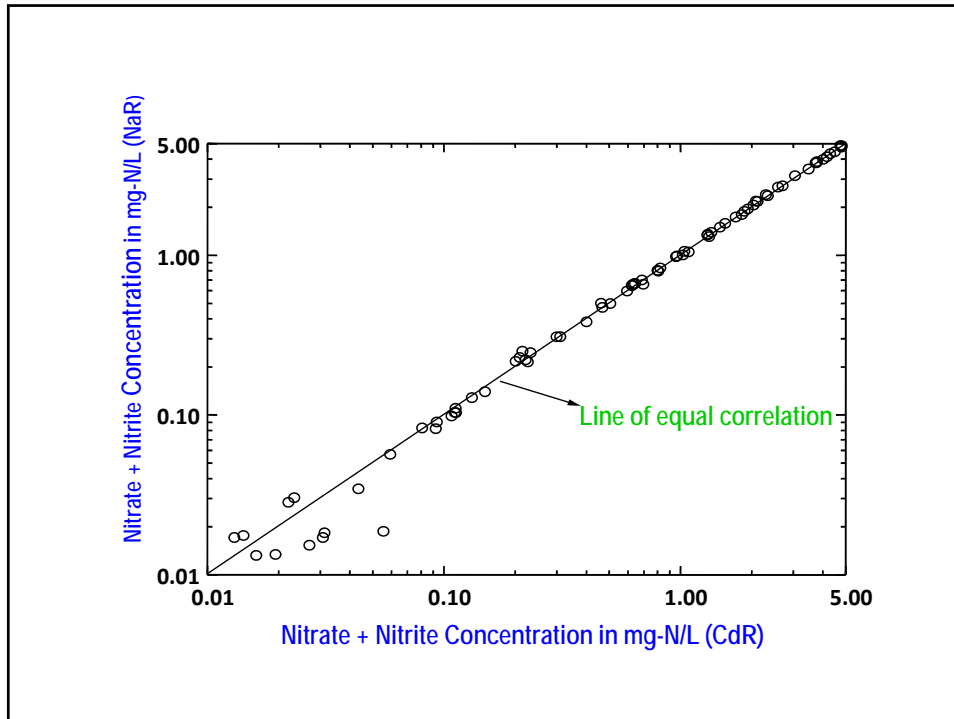
NaR and **NADH** replace the toxic **cadmium** reagents used in conventional nitrate analysis methods.



Minimal change required from standard methods

3-D Model of Nitrate Reductase





Enzyme-based Nitrate Method Validations

- **ASTM D7781** for *all* water types (potable to waste water effluents)
- **US EPA ATP Case No. N07-0003** for all Clean Water Act applications (everything except drinking water) using autoanalyzers
- **US EPA ATP** published in the Methods Update Rule (MUR) 20 Feb 2015
- **USGS I-2547-11** and **I-2548-11**
- **Federal Register (40CFR136)** by **December 2015**
- Working on **Std Methods** and **EPA SDWA now**



NECi Nitrate Test Products

- AtNaR and YNaR vials and Reagent Packs
- Test tube format kits for Labs with pipets, photometers, & ability to handle acids
- Microplate format
- Reagent packs for specific instruments:
 - Discrete Analyzers
 - Flow Injection Autoanalyzers
- SIMPLIFIED nitrate test kits (NTKs) for agricultural sample types:
 - Water
 - Soil
 - Forage



Accuracy

Plasticware is accurate to $\pm 2.0\%$ or less.

Dilution factors are built into the kits. This is how the kits accommodate different sample types.

Soil scoop holds approx 1 gram of soil. Variation for soil type and moisture is less than 10%.

Protocols for drying to constant weight are available upon request.

Wet versus dry feeds: Forage, silage, green chop, etc average 50% moisture. We account for this factor in the color charts and nitrate standards used for Livestock Feed kits. Wet feed values are reproducible.



NECi's Ag NTKs

Sampling

- The accuracy of information from any testing regimen depends in part on where, how, and when you take your samples.
- We recommend following the advice of your technical service provider for best results.
- Ag NTKs are best when you need immediate information, for spot checks and to test known trouble spots.



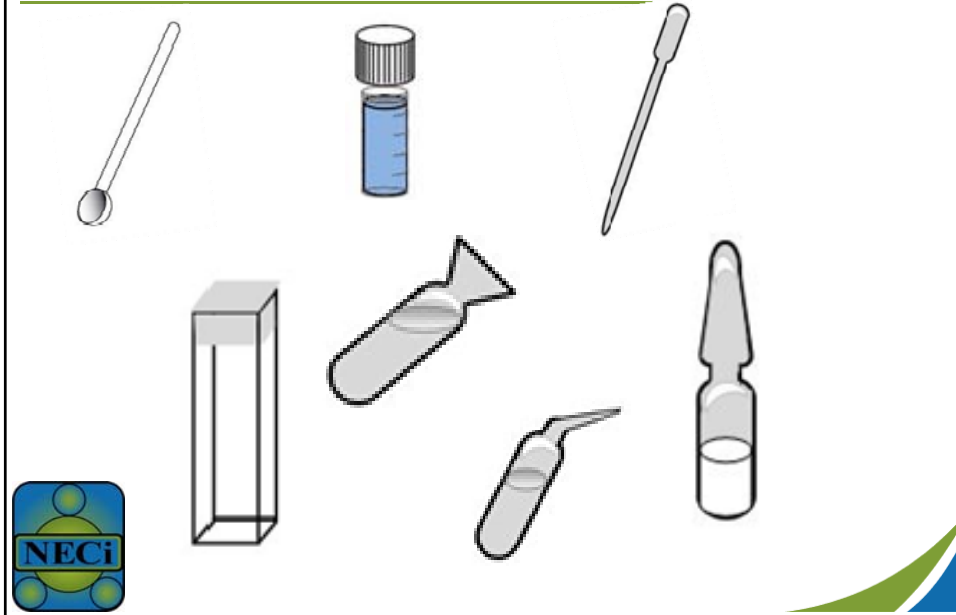
Sample Preparation

NECi's Philosophy: Simple is Better

- **Sample size is small in relation to assay volume: generally 20-fold dilution**
- **Water soluble extraction only: for most sample types, especially soil**
 - Filtration or centrifugation when necessary
 - Improved reproducibility
 - Measure N or P available to roots
- **Less handling = fewer chances to introduce error: in protocol or calculations**



Soil Nitrate Test Kit



Lab Kit vs NTK, ppm Nitrate-N in Soils

Soil source	LAB	NTK-S
Under a bird-feeding area	>100ppm	blowout
Same, diluted 1:4	130.98	130.1
Neglected flower bed	1.22	nondetect
Residential backyard	1.47	nondetect
School demo vegetable garden	30.51	28.0



Samples tested for Validation Studies

Sample Type	Filtered	Acid
Denver area treatment plant Influent wastewater	Yes	Yes
Denver area treatment plant Wastewater effluent #1	Yes	Yes
Denver area treatment plant Wastewater effluent #2	Yes	Yes
Michigan paper mill waste stream effluent	Yes	Yes
Denver area metal finisher waste stream effluent	Yes	Yes
Denver area Commercial laundry waste stream effluent	Yes	Yes
Environmental Resources Associates #507 Hardness WasteWatR reference material	Yes	Yes
Michigan Confined Animal Feeding Operation (CAFO) effluent from tiled field	Yes	Yes
Low-nutrient seawater (collected offshore Hawaii)	Yes	No
ERA # 608 Reference Standard	Yes	Yes
USGS PE N-116 (low nutrient-fortified river water)	Yes	No
USGS PE N-115 (high nutrient-fortified river water)	Yes	No
Tap water at each lab		
Tap water plus added Chlorine		

Table 4: Enzymatic Reduction Efficiency Summary

Acceptance Standard is 90% or greater reduction efficiency.

2nd Source = Nitrate Standard in mg N/L Nitrite Standard = mg N/L

		A-540 nm	mg N/L	Reduction Efficiency
Lab 1	2nd Source	2.50	0.35217	102.1%
	Nitrite Std	2.50	0.34507	
Lab 2	2nd Source	2.50	0.36664	101.3%
	Nitrite Std	2.50	0.36190	
Lab 3	2nd Source	2.50	0.2456	107.2%
	Nitrite Std	2.50	0.2292	
Lab 4	2nd Source	2.50	0.295	116.6%
	Nitrite Std	2.50	0.252	
Lab 5	2nd Source	2.50	0.33422	94.9%
	Nitrite Std	2.50	0.3514	
Lab 6	2nd Source	2.50	0.37078	103.8%
	Nitrite Std	2.50	0.35867	
Lab 7	2nd Source	2.50		103.7%
	Nitrite Std	2.50		
Lab 8	2nd Source	2.50	0.358	102.3%
	Nitrite Std	2.50	0.350	
Lab 9* [550nm]	2nd Source	3.04	0.2127	98.6%
	Nitrite Std	3.04	0.2151	
Lab 10	2nd Source	2.50	0.146	105.0%
	Nitrite Std	2.50	0.140	

**Table 5 from EPA Validation Report:
Initial Performance and Recovery (IPR) Summary**

Lab	# Analyses	Mean Recovery (%)	RSD (%)	Minimum Recovery (%)	Max Recovery (%)
1	4	100.67	0.71	99.67	101.30
2	4	101.82	0.54	101.29	102.40
3	4	96.16	2.23	93.50	98.70
4	4	106.38	0.73	105.82	107.52
5	4	101.79	0.78	100.63	102.37
6	4	102.76	0.92	101.77	103.93
7	4	100.50	1.13	98.97	101.66
8	4	98.45	0.66	97.77	99.21
9	4	101.11	0.95	100.17	102.43
10	4	99.34	2.45	95.85	101.50

Table 7: Method Detection Limit (MDL) Summary

Abbreviations:

DA = Discrete Analyzer; NA = Not Analyzed.

**One replicate discarded due to issue with blank*

Lab	Method	MDL	Replicates	Spike	Spike/MDL
		mg N/L		mg N/L	Ratio
1	DA	0.0079	8	0.040	5.068
2	DA	0.0148	8 (7)*	0.040	2.701
3	DA	0.0130	7	0.050	3.832
4	DA	0.0055	7	0.025	4.522
5	DA	0.0226	7	0.050	2.215
6	DA	0.0310	7	0.050	1.615
6	DA	0.0260	7	0.075	2.881
8	DA	NA			
9	DA	0.0060	7	0.045	7.541
10	DA	0.0463	7	0.100	2.160

PO4 Standard Curve 0-5 PPM Pi			
Sample/Std (ppm)	Abs @360 nm	Avg Abs	Abs less Blank
0 (Reagent Blank)	0.155	0.154	0.000
	0.149		
	0.157		
0.5	0.182	0.182	0.028
	0.183		
	0.181		
1	0.214	0.215	0.062
	0.220		
	0.213		
2	0.283	0.280	0.126
	0.278		
	0.278		
3	0.345	0.345	0.191
	0.345		
	0.344		
4	0.410	0.409	0.255
	0.410		
	0.408		
5	0.464	0.464	0.310
	0.459		
	0.468		

NECi's Photometer ties it together: Biotechnology People can Use!

Nitrate test results are pink, but Phosphate results are in UV, not visible to the eye. And most users want to see a NUMBER: results should be in digital format.

So we are introducing our own nitrate/phosphate photometer. Smartphone based with data exportable to Excel. Beta testing of prototypes ongoing thru Summer 2015.



Enzyme Reagents Under Development

Under development now:

- Total N using NECi's NaR
- Total P using NECi's PNP
- Glycerol
- Ammonia
- Galactose
- Ethanol in beverages and fuel

NECi's new N & P Photometer

Inexpensive LED-based
device with interface to
Smartphone or Laptop

