Adventures in Citizen Science

Reagent Grade Enzymes and Open Source Lab Equipment

Ellen R. Campbell, CEO
Wilbur H (Bill) Campbell, PhD

CITIZEN SCIENTISTS NEED ACCURATE DATA

- Why gather data that is not accurate, reliable, and precise?
- Why use legacy methods that require hazardous reagents and large volumes?
- It's the 21st Century! We need data in digital, exportable formats.
- Accurate optical data requires portable photometers that accept standard 1 mL flat-sided cuvettes. Affordable device needed.

We've combined biotech with an inexpensive photometer to enable *sustainable science*.

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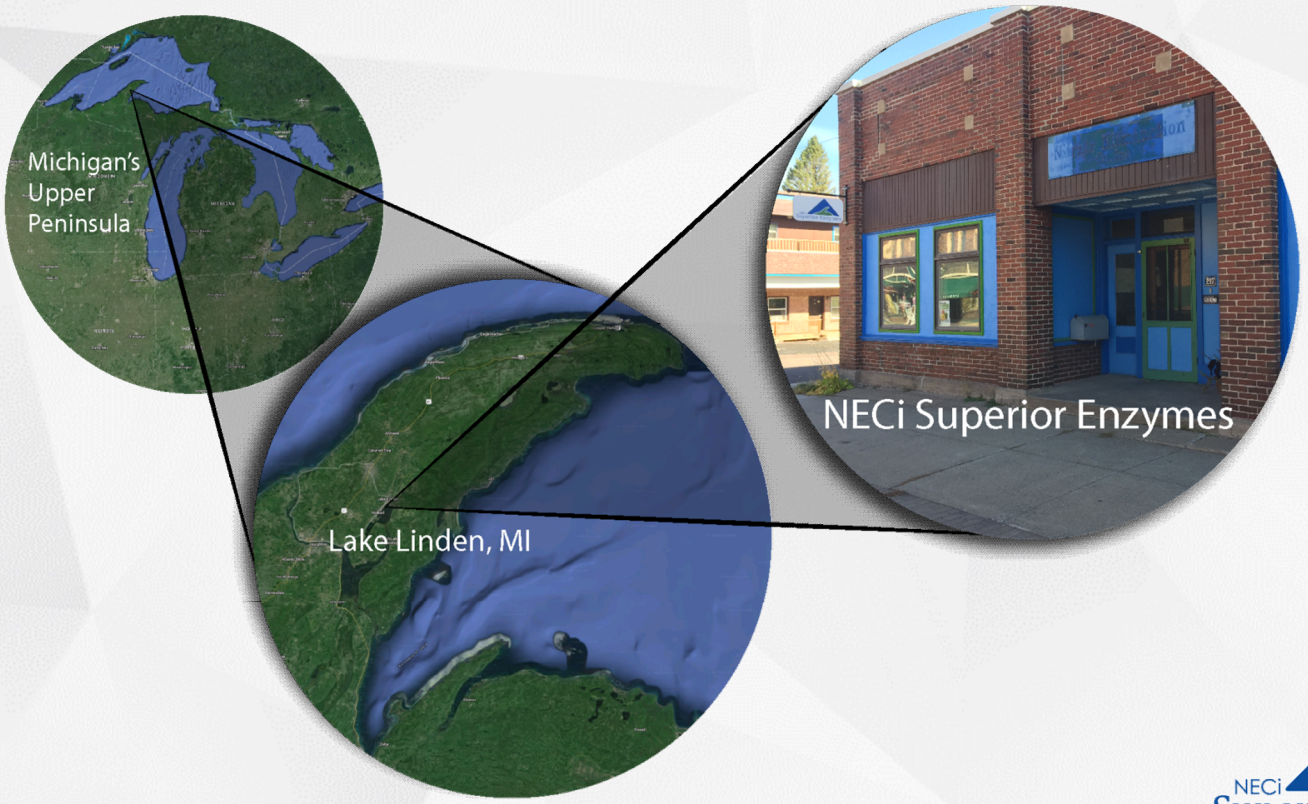

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REAGENT GRADE ENZYMES FOR CHEMISTRY

- **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

Enzyme-based analytical chemistry is accurate, reliable, and environmentally benign.

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FROM BASIC RESEARCH TO CITIZEN SCIENCE

- **How do plants use nitrogen fertilizers?**
- **How does Nitrate Reductase (NaR) help plants make proteins and DNA?**
- **Led to investigation into enzyme structure and function**
- **Development of MAbs to enable purification**
- **Early studies in molecular biology (1980s)**
- **Development of nitrate assay using purified NaR (1988)**
- **Enzyme-based nitrate removal system published in *Nature* (1992)**

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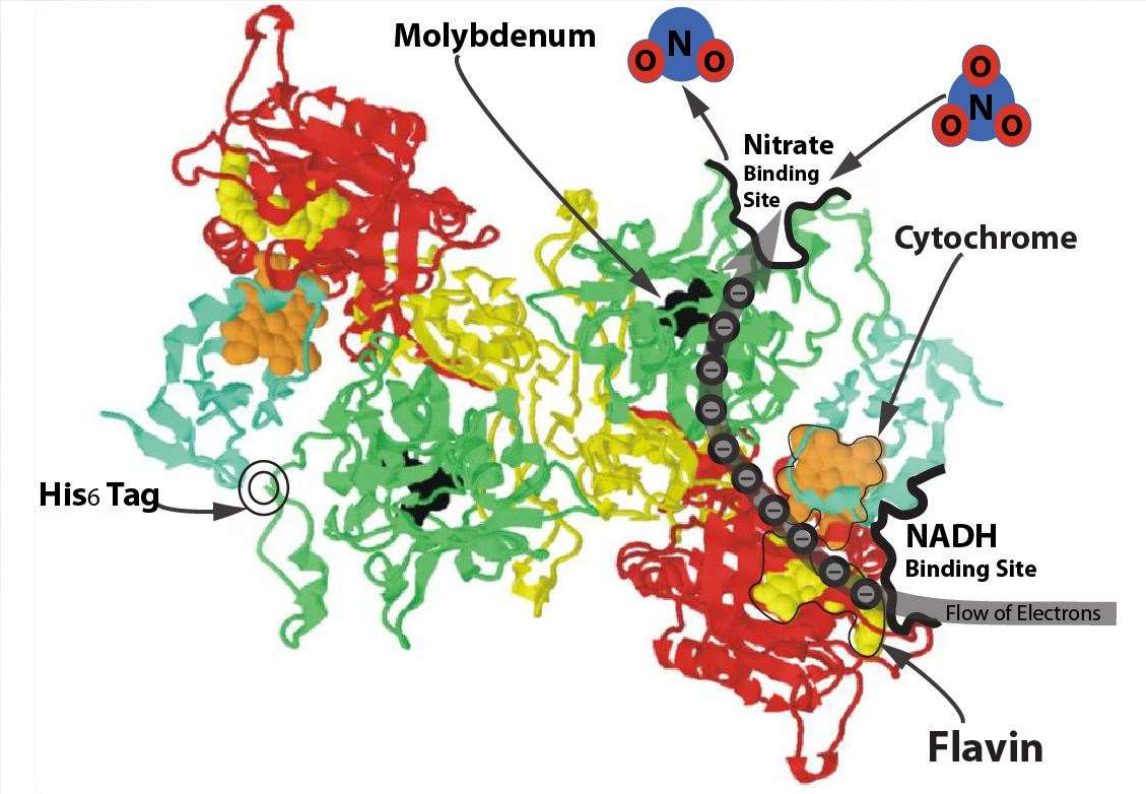
NITRATE REDUCTASE



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ENZYME BASED NITRATE ANALYSIS

- **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 or zinc reagents used in conventional nitrate analysis methods.**

Minimal change is required from standard methods

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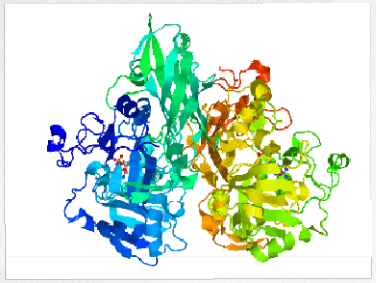

$NO_3 + NADH + H^+ \xrightarrow{\text{Nitrate Reductase}} NO_2 + NAD^+ + H_2O$

NO_2 + Sulfanilic Acid ⟶ Diazonium Salt

Diazonium Salt + NED ⟶ Pink dye for measuring A 540 ± 20nm

Samples are measured against a standard nitrate curve to determine results in ppm Nitrate-N

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From Basic Research to Products

- NECi founded in 1993
- R&D funded by the Small Business Innovation program
- NIH funded cloning and expression of NaR
- USDA funded product development: lab reagents and test kits
- NSF funded development of the Photometer
- USDA funded expression and test kits for Phosphate
- NECi's nitrate detection reagents on the market since 1993. Recombinant NaR since 2001.

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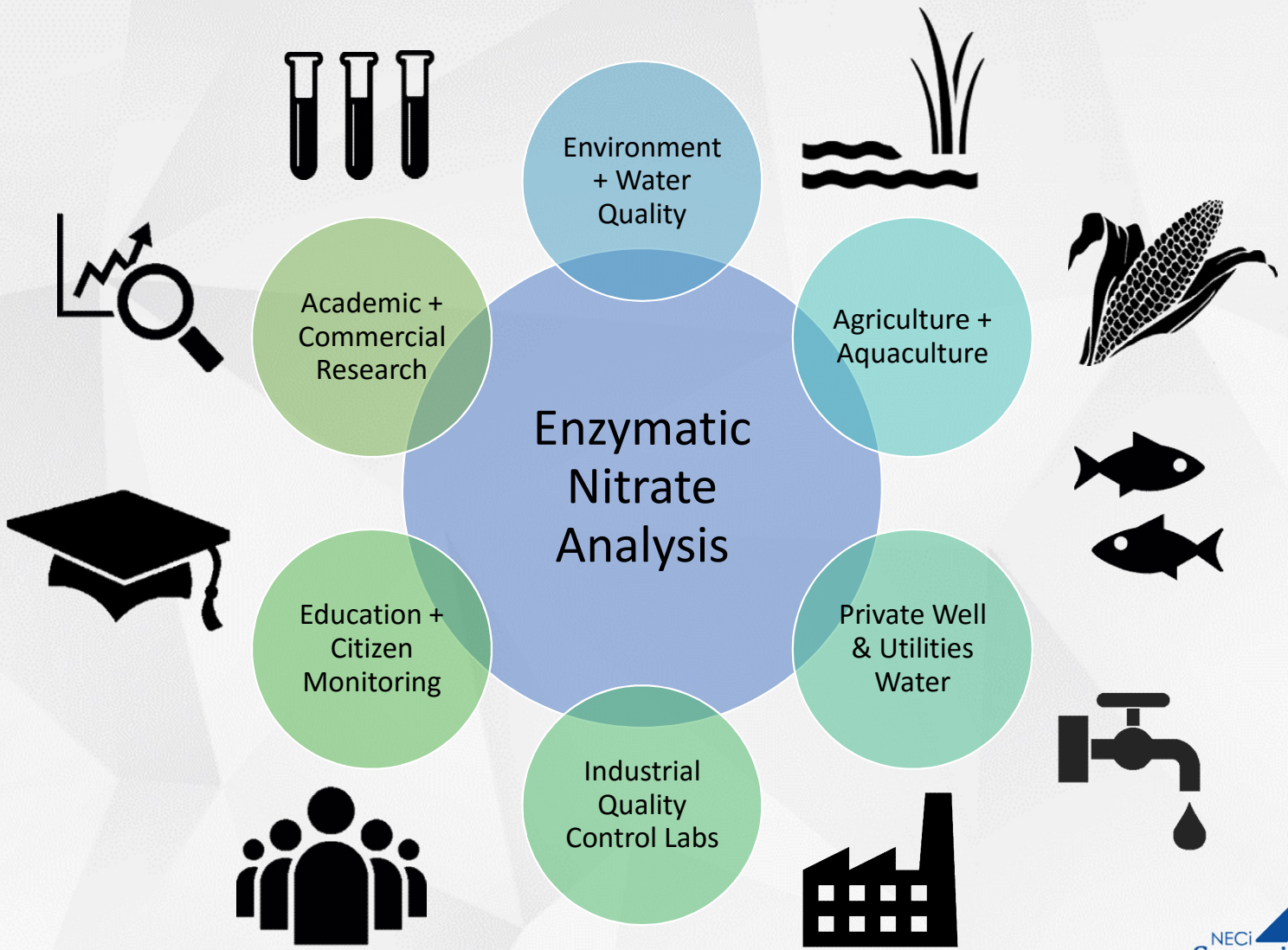
Enzymatic Nitrate Analysis
Environment + Water Quality
Agriculture + Aquaculture
Private Well & Utilities Water
Industrial Quality Control Labs
Education + Citizen Monitoring
Academic + Commercial Research
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BENEFITS OF RECOMBINANT NITRATE REDUCTASE

- **Recombinant enzyme** design & production
  - Stringent quality control, specified traits & batch consistency
- **Nitrate reductase simply replaces cadmium**
  - Griess reaction follows nitrate reduction = minimal training to switch
- **Active site** only binds nitrate
  - High specificity, low sensitivity, rare interferences in complex matrices
- **Nitrate reductase** naturally occurring enzyme in plants
  - Non-toxic for lab personnel + no hazardous shipping or waste fees

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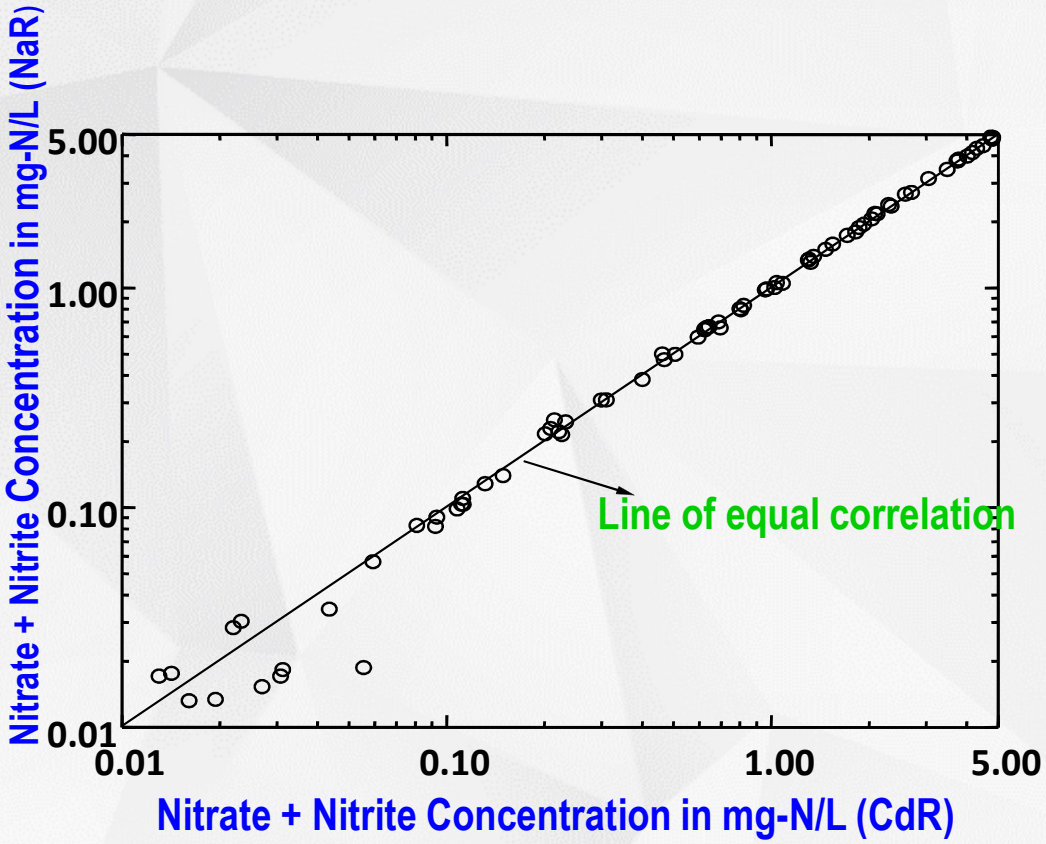
USGS VALIDATION: ENZYME VS. CADMIUM



Nitrate by NaR vs nitrate by Cadmium.

Data courtesy of USGS in Denver CO

Groundwater samples analyzed by Discrete Analyzer

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USGS VALIDATION: ENZYME VS. CADMIUM

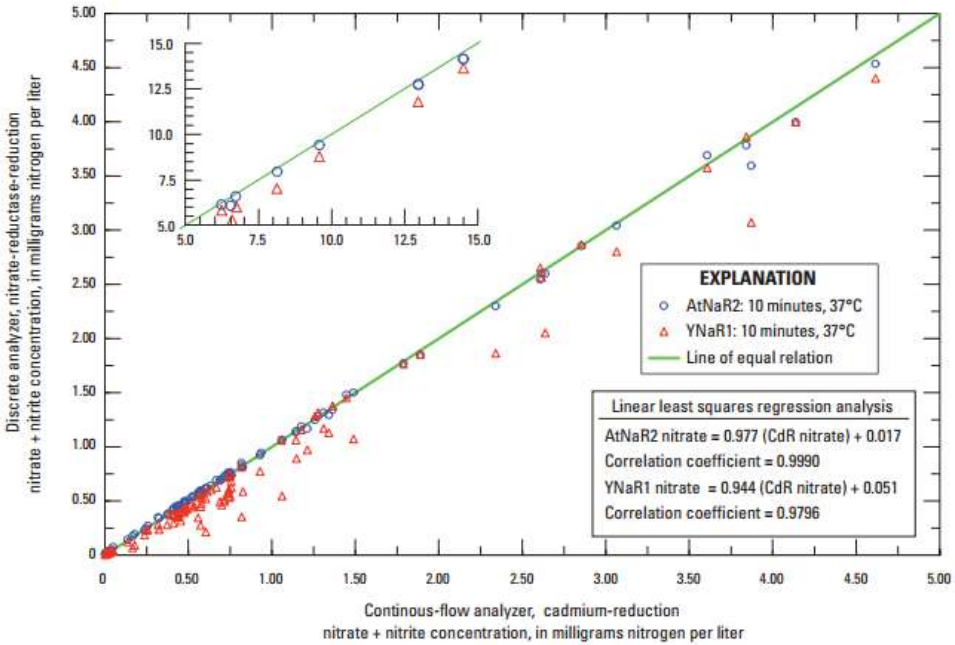

Figure 12. Comparison of nitrate + nitrite concentrations for 115 environmental water samples analyzed by automated continuous-flow, cadmium-reduction (CdR) method and automated discrete analyzer (DA) enzymatic-reduction method with NAD(P)H:YNaR1 nitrate reductase and NADH:AtNaR2 nitrate reductase. DA determinations were consecutive on November 9, 2005.

Source: Colorimetric Determination of Nitrate Plus Nitrite in Water by Enzymatic Reduction, Automated Discrete Analyzer Methods. USGS 2011. http://pubs.usgs.gov/tm/05b08/contents/TM5-B8.pdf

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Enzymes for simple yet accurate Test Kits

- **Start with common validated lab methods**
- **Develop appropriate enzyme and assay**
- **Simplify lab methods for ease of use**
- **Simple extraction with water as solvent**
  - ***Measure available nitrate and phosphate***
- **Sample size is small in relation to assay volume**
  - ***Generally 20-fold dilution to minimize interferences***
- **Less handling = fewer chances to introduce error**
  - ***In protocol or calculations***

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WHICH ONE WILL YIELD BETTER DATA?

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ENZYMATIC TEST KITS FOR CITIZEN SCIENCE

- **Fast** Results in 20 minutes
- **Easy** Prepackaged, premeasured, pre-calculated
- **Versatile** Nitrate (and phosphate) in many sample types
- **Accurate** Strong correlation to laboratory data
- **Economical** ~ $5 per sample (varies with test kit size)
- **Safe** Non-toxic reagents for handling & disposal

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VARIOUS SAMPLE TYPES

- Well + Ag Water
  - Standard Range Levels
- Environmental Water
  - Low Range Levels
- Soil
- Plant Petioles
- Green or Dry Animal Forage

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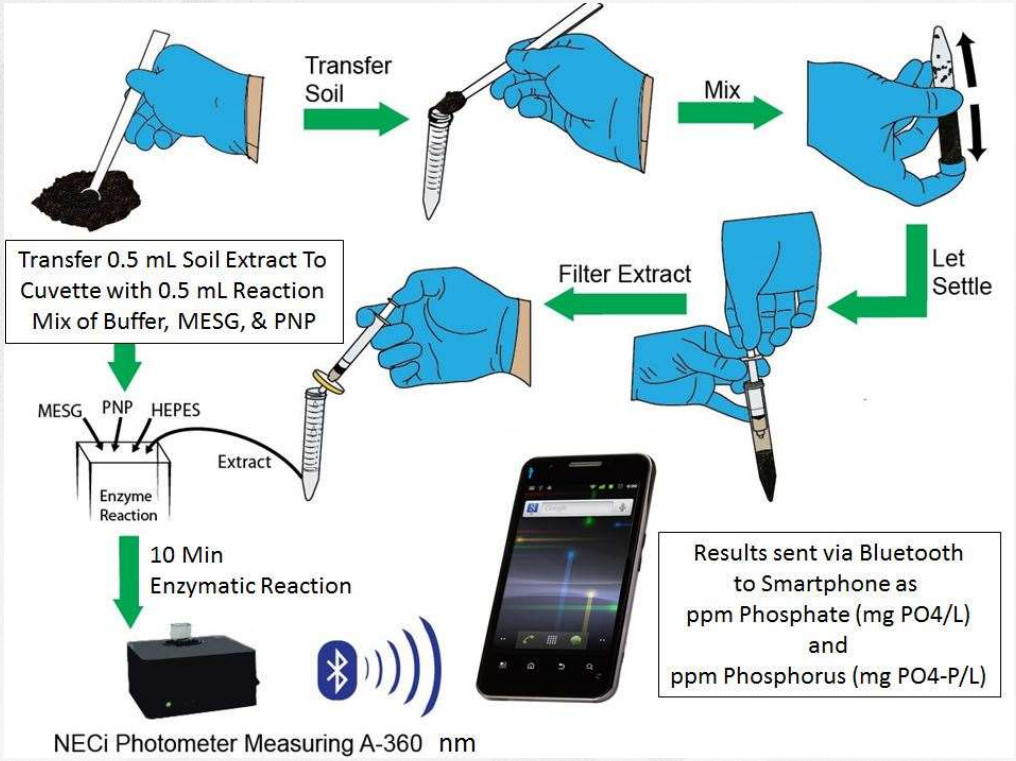
ENZYME BASED PHOSPHATE TEST KITS



- Methods X Publication April, 2015
- Project funded by U.S. Dept. of Agriculture
- Test kits now available for soil, low and standard range water, and laboratory analysis

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21st CENTURY CITIZEN SCIENCE TECHNOLOGY

Legacy Technology + Test Kits	NECi Photometer + Test Kits
Add Reagents to 5-10 mL Sample	50 µL Sample into Buffer
Cadmium or Zinc Powder	Freeze-dried Enzyme
Round Glass Vials	Flat-Sided Cuvettes
2+ Points of Manual Data Entry/Transfer	Automated Data Input, Storage, Transfer
Acidification or Complex Preparation	Water Extraction Sample Preparation
Manual Sample Detail Entry	Sample Collection Time + GPS Stamp
Bulky, Expensive Equipment	Low-Cost, Handheld Instrument

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NSF FUNDED OPEN-SOURCE PHOTOMETER

- **Collaborative Effort with Michigan Technological University**
- **Open Source 3-D Printed Case Design by Dr. Joshua Pierce's Graduate Team**
- **SBIR/STTR program award #IIP-1417061**
- **$65 in materials to build your own**
- **PLOS One publication summer 2015**

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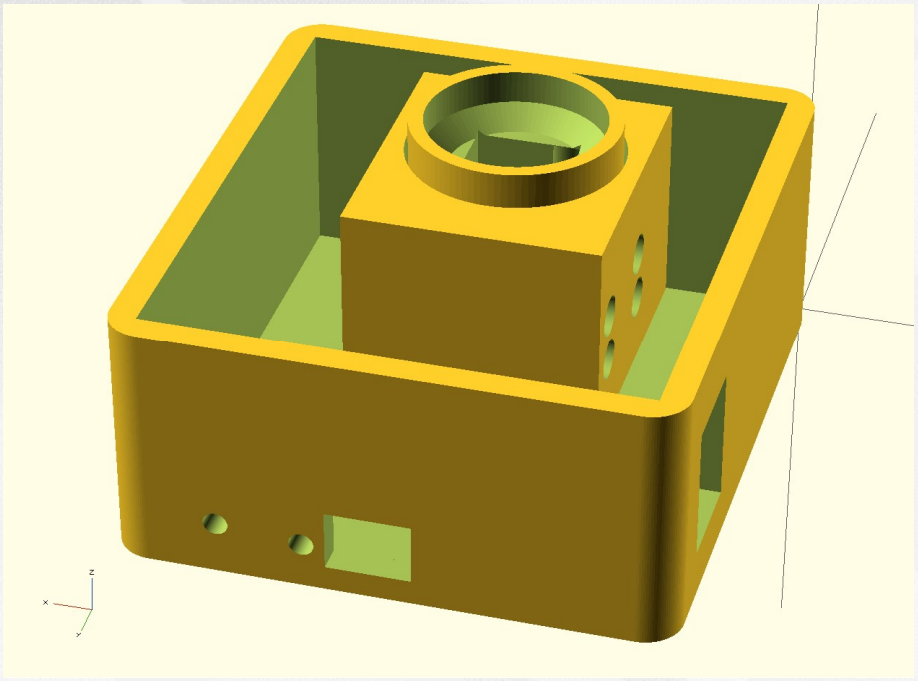
SQUARE OPTICS ARE BETTER THAN ROUND!



NECi photometer optical bench

Uses Standard and micro 1 cm cuvettes

Double beam for accurate blanking and background

Dual wavelength LEDs for versatility and low power requirements

Design features lead to increased accuracy

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COMMERCIAL HANDHELD PHOTOMETER

- **Dual Wavelength for reliable nitrate and phosphate data anywhere**
- **Data is stored and tracked on Android devices for export and further analysis**
- **User-friendly, non-toxic test kits with improved mobile app interface**
- **Applications for alcohol & glycerol in biodiesel, fuel, and beverages in progress**

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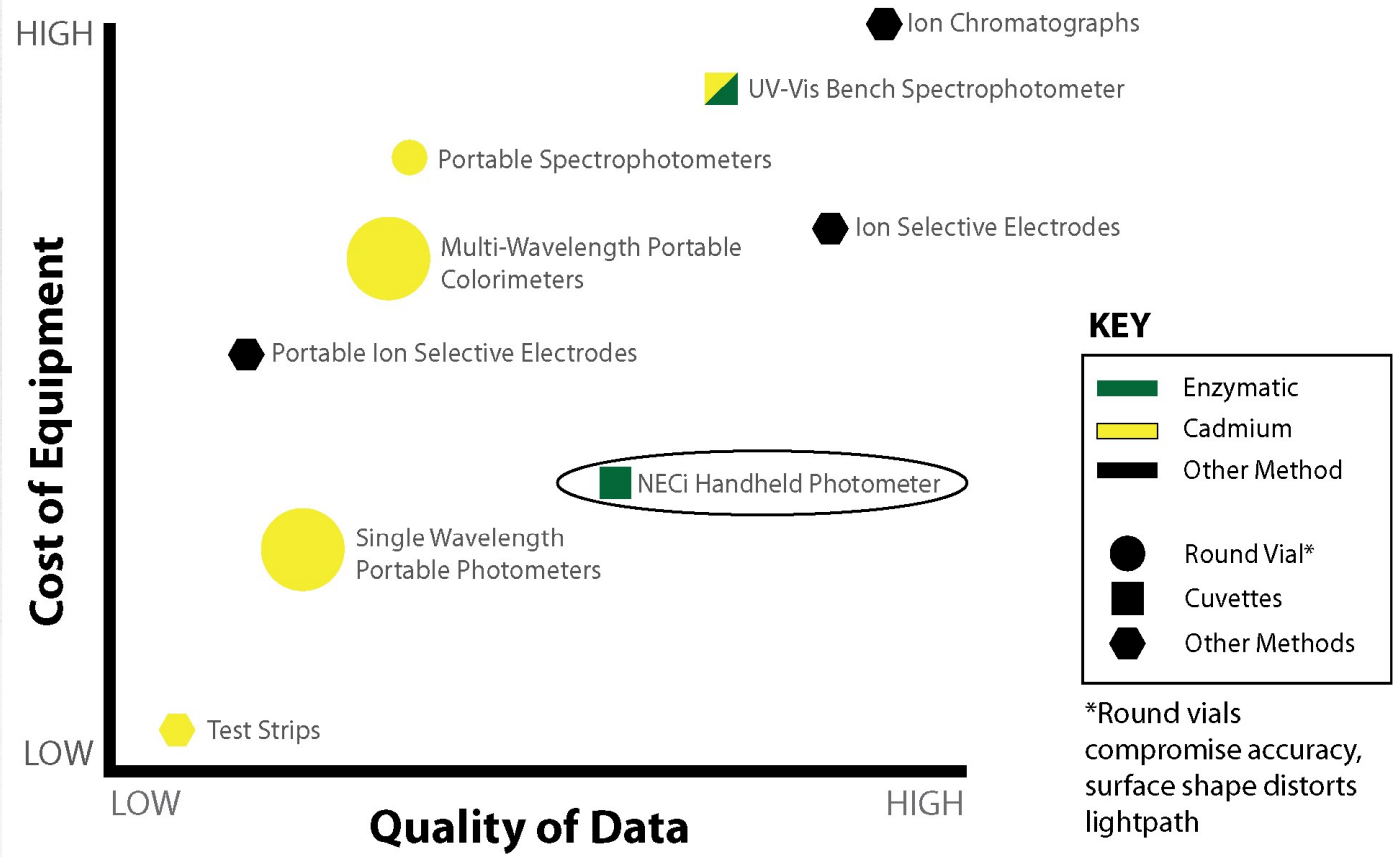
Nitrate Analysis Equipment: Cost vs. Data Quality



*Round vials compromise accuracy, surface shape distorts lightpath

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METHOD VALIDATION DATA

Interagency round-robin studies showing accuracy + reliability of nitrate reductase method for nitrate-n analysis

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EPA CLEAN WATER ACT ATP SAMPLE MATRICES

Sample Type	NaR Reduction (mg N/L)	Cd Reduction (mg N/L)
Denver area treatment plant Influent wastewater	**0.03**	**0.03**
Denver area treatment plant Wastewater effluent #1	**7.8**	**7.6**
Denver Area treatment plant Wastewater effluent #2	**0.23**	**0.26**
Michigan paper mill waste stream effluent	**0.04**	**0.03**
Denver area metal finisher waste stream effluent	**270.8**	**272.6**
Denver area Commercial laundry waste stream effluent	**4.8**	**4.8**
Environmental Resources Associates #507 Hardness WasteWatR reference material	**0.05**	**0.06**
Michigan Confined Animal Feeding Operation (CAFO) effluent from tiled field	**13.77**	**14.1**
Low-nutrient seawater (collected offshore Hawaii)	**0.027**	**0.030**
ERA #608 Reference Standard	**6.8**	**7.02**
USGS PE N-116 (low nutrient-fortified river water)	**0.45**	**0.48**
USGS PE N-115 (high nutrient-fortified river water)	**2.28**	**2.36**

Source: U.S. EPA Alternate Test Procedure Validation Report for Enzymatic Reduction Method of Nitrate-N Analysis. Data Merged from Tables 1 + 11.

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CLEAN WATER ACT EPA VALIDATION STUDY

Table 5 Initial Performance and Recovery (IPR) Summary

Lab	# Analyses	Spike Conc. (mg/L)	Mean Recovery (%)	RSD (%)	Minimum Recovery (%)	Maximum Recovery (%)
2	4	2.5	100.67	0.71	99.67	101.30
3	4	2.5	101.82	0.54	101.29	102.40
4	4	2.0	96.16	2.23	93.50	98.70
5	4	2.5	106.38	0.73	105.82	107.52
7	4	2.0	101.79	0.78	100.63	102.37
8	4	2.5	102.76	0.92	101.77	103.93
9	4	2.0	100.50	1.13	98.97	101.66
10	4	2.0	98.45	0.66	97.77	99.21
11	4	2.7	101.11	0.95	100.17	102.43
12	4	2.0	99.34	2.45	95.85	101.50

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ADDITIONAL ANALYTES

- **We've adapted the photometer for alternative wavelengths.**
  - **Soil health: microbial respiration test**
  - **Turbidity**
- **We've developed recombinant forms of reagent grade enzymes for new analytes and sample types**
  - **Glycerol in biodiesel for fuel QA/QC**
  - **Ethanol**
  - **Galactose and other sugars for food QA/QC**
  - **Peroxide**

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NECi Superior Enzymes Thanks the Following for Their Work and Support:

Small Business Innovation Research programs of
The US Dept of Agriculture (nitrate and phosphate reagents and test kits)
The National Science Foundation (photometer)

- **Michigan's ETF program (travel to conferences)**
- **Validation partners at ASTM International, US EPA, & US Geological Survey**

The people who produced this work:

- **Dr JM Pearce and students at MI Technological University**
- **Dr CJ Patton, USGS in Denver, CO**
- **NECi Scientists and Technicians**

Find more information at www.nitrate.com

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