Rapid and Efficient Extraction Method of Chlorophyll by Bead Beating

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PROJECT CLEAN LAKE
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Organization of Presentation

- What is Chlorophyll?
- Why Test For It?
- Types of Algae
- Detection Method (Method 445)
- Extraction method
- Alternative extraction method
- Statistical comparison of the extraction
- Conclusion

Chlorophyll a

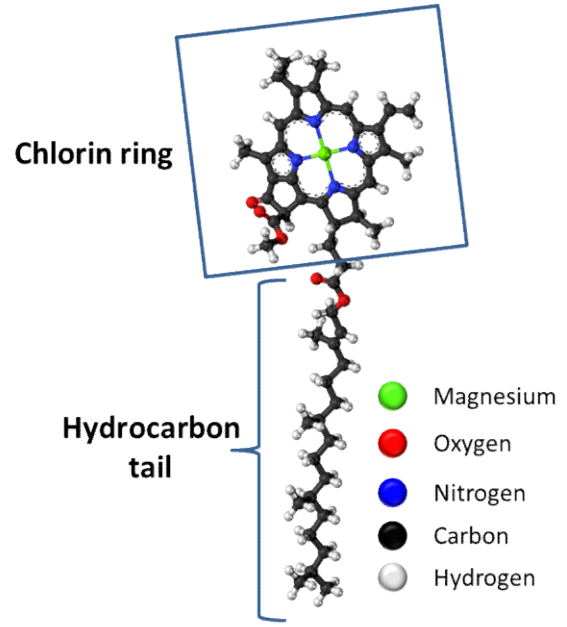
- Chlorophyll is a Mg^{2+} based green pigment that harvests sunlight and uses water and carbon dioxide into glucose, found in plants, algae and phytoplankton.
- There are six different chlorophyll molecules that have been identified: A, B, C1, C2, D & F. Most labs focus on the most common type, A.
- Pheophytin is a chlorophyll without the Mg^{2+} .
- Chlorophyll A is the primary molecule responsible for photosynthesis in all photosynthesizing organisms, from plants to algae and cyanobacteria.

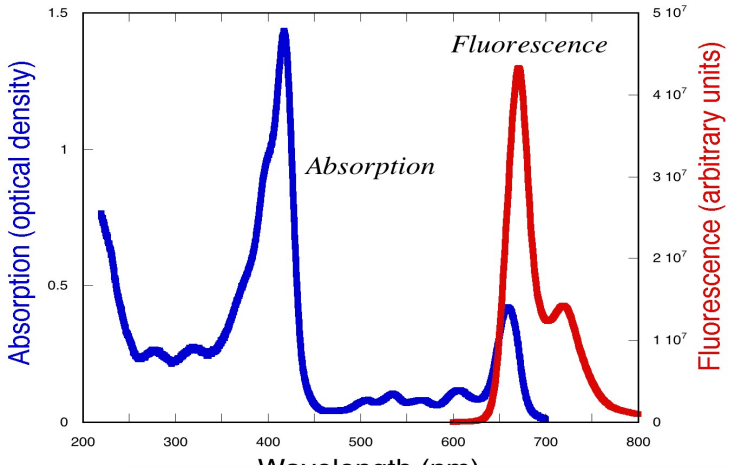
Structure of Chlorophyll a

- The structure of chlorophyll *a* consists of a chlorin ring, surrounding a Mg^{2+}, and has several other attached side chains and a hydrocarbon tail.

- The fluorescence of chlorophyll varies upon the solvents

- In 90 % Acetone we estimate the Chlorophyll a using Turner Trilogy spectrofluorometer using the excitation wavelength of 485 nm and emmission wavelength 685 nm.





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Chlorophyll and Water quality

- Degraded water quality condition is indicated by increased of algal biomass and can be measured by the concentration of chlorophyll a.
- High algal presence cause aesthetic problems such as green scums and bad odors, and can result in decreased levels of dissolved oxygen.
- Waters with high levels of nutrients from fertilizers, septic systems, sewage treatment plants and urban runoff have high concentrations of chlorophyll a and excess amounts of algae.
- Determination of chlorophyll content has been routinely applied for determination of algal and phytoplankton levels in marine and freshwater to assess the eutrophic status and thereby monitor water quality.

Why are chlorophyll A levels important?

- Nutrient level indicator
  - Chlorophyll A levels increase with nutrient levels
- Indicates water clarity – total suspended solids, turbidity
- Chlorophyll A is half of the story
  - Different nutrient levels will support different types of algae.
  - Different Algae will support different aquatic consumers
- Harmful Algal Blooms
  - Toxic algae
  - Oxygen depletion
  - Aesthetics and increased bacteria levels at beaches

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Lake Erie Has Algae

- Is this Fish food or Fish poison

- Chlorophyll A alone will not tell us

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Toxic Algae Can Be a Public Hazard

- We have all heard of fish kills caused by cyanobacteria
- Every year, hunting dogs die from water that contained cyanotoxins.
- In 2002, two boys in Madison, WI died after swimming in, and ingesting water from a golf course pond.
- Non toxic algal blooms also promote bacteria growth, reduce water quality, and discourage beach use.

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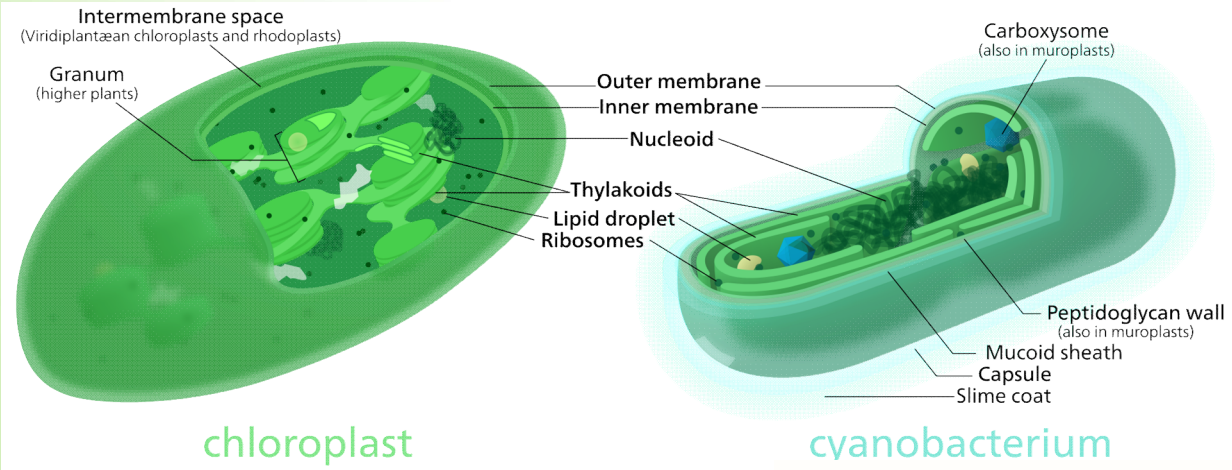
Chlorophyll and HABS

- Chlorophyll levels in the water bodies are used for several predictive models of Harmful Algal Blooms (HABs) caused by cyanobacteria
- Interestingly cyanobacteria are considered to be the ancestors of chloroplasts. They are sometimes called blue-green algae even though they are prokaryotes
- Like chloroplasts, they have thylakoids including chlorophyll *a*



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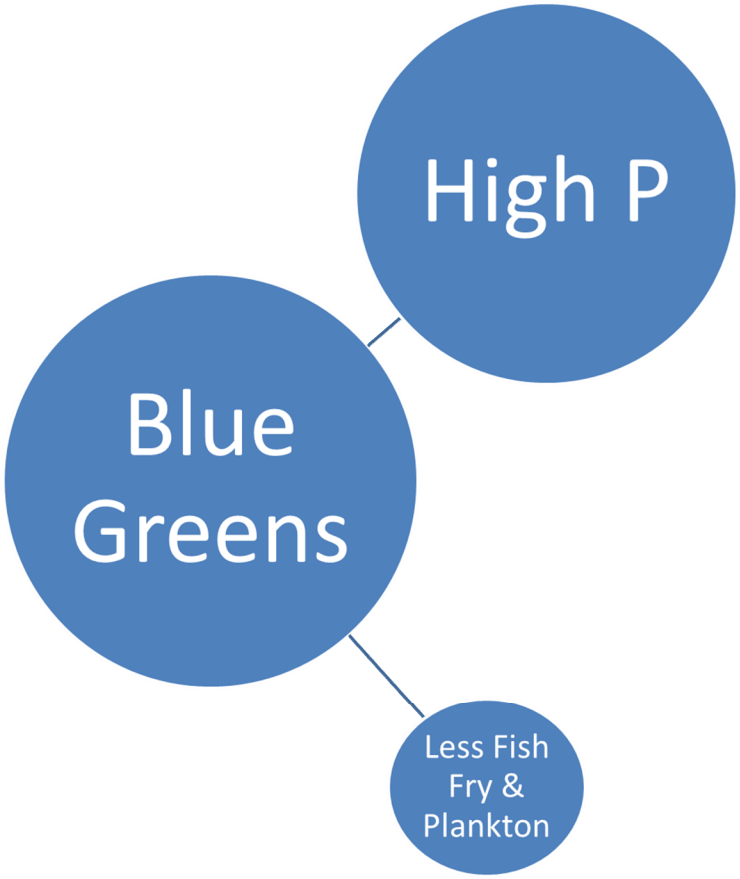
How Phosphorus Affects the Aquatic Food Web



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Equipment for Method 445

- Turner trilogy Fluorometer
- Tissue Grinder
- Centrifuge
- Dark room
- 47 mm glass fiber filters
- Forceps
- Filtration manifold
- Filtration funnels and bases
- 15 mL conical tubes
- Volumetric or auto-pipettes w/tips
- Aluminum foil
- Freezer (-30°C)

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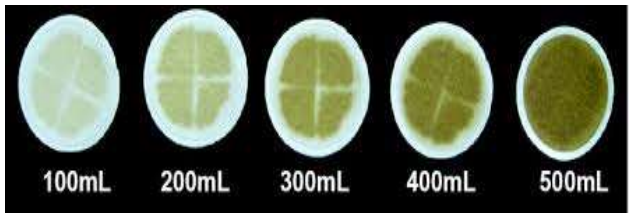
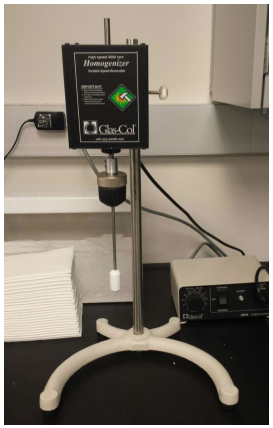
Reagents and Standards

- 90% acetone
- DI water
- Standards
  - Calibration standards
  - ICV – independent calibration verification
  - CCV – continuing calibration verification
  - CCB – continuing calibration blank

Method 445.0 workflow

- **Sample Collection:** 1 L to 4 L water sample by grab or pump sampler
- **Filtration:** 100 ml of the sample is vacuum filtered through a glass fiber filter (0.7µ), 3 replicates per sample
- **Preservation:** The filters can be preserved for 3.5 weeks at –20° C to –70° C.
- **Extraction:** The chlorophyll a in the filters is extracted by grinding the filters in 90% acetone.





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Method 445.0 workflow (Contd.)

- **Steeping:** The extracted samples are then steeped at 4° C for 2 h to 24 h.
- **Centrifugation:** The tubes are then centrifuged at 675 g for 15 minutes.
- **Fluorometric analysis:** The supernatant is then read on the Turner Trilogy fluorometer to determine the chlorophyll concentration

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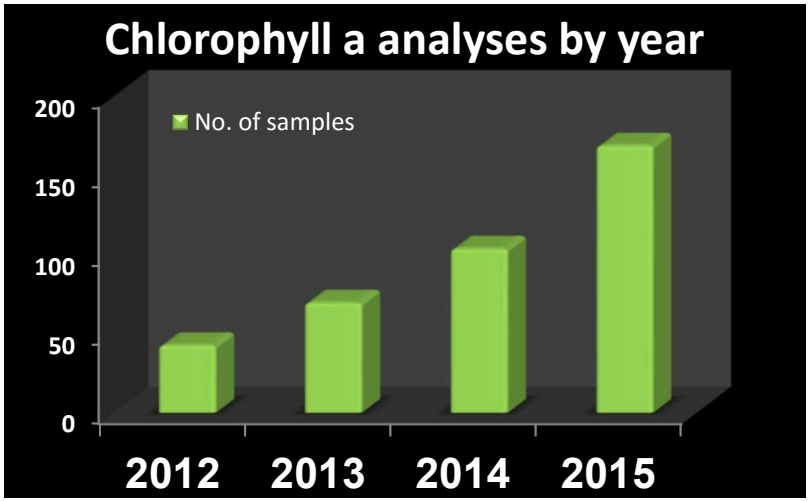
Chlorophyll a measured at NEORSD

- At NEORSD we routinely monitor the chlorophyll a levels of Lake Erie, the Cuyahoga River and its tributaries.
- We used Method 445.0 for the *In vitro* determination of chlorophyll a in marine and freshwater algae by fluorescence.
- Method 445.0 is used to detect low levels of chlorophyll a (chl a) and pheophytin a (pheo a

YEAR	NO. OF SAMPLES
2015	169
2014	104
2013	70
2012	43



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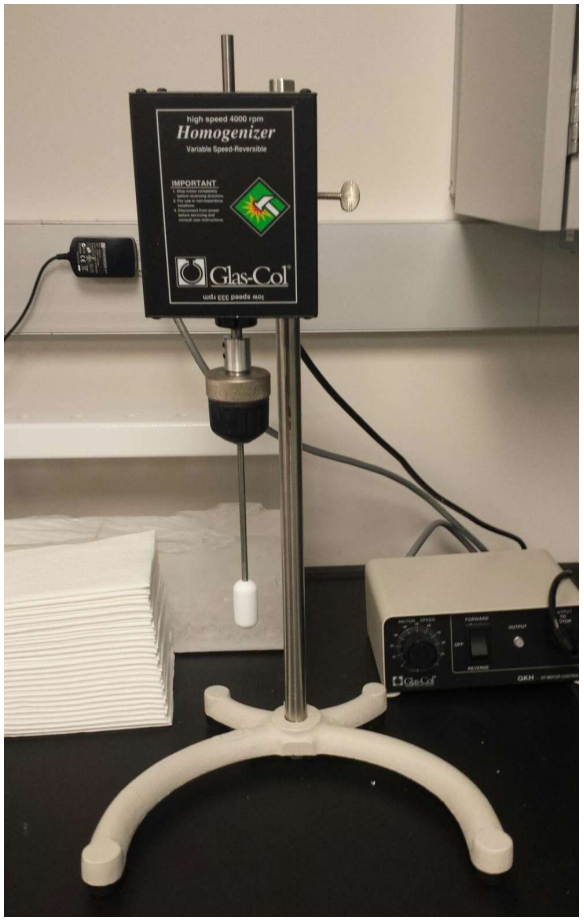
Extraction by homogenization

- The extraction of chlorophyll from the filters was done by masticating the filters in a glass tube with 10 ml of 90% acetone using a Teflon pestle attached to a homogenizer.



DRAWBACK:

- This method allows processing of only two samples (in triplicates) in **20 minutes** i.e. about 3 minutes per glass fiber filter.
- Loss of samples while transfer the crushed filters

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Alternative extraction method

- The new method of extraction involves Bead Ruptor 24.
- The filters are placed in 35 ml tubes containing 2.8 mm ceramic beads
- Beated at 2.6 m/s speed for 1 minute, carefully monitoring the temperature
- This enables two samples to be extracted in just 1 minute

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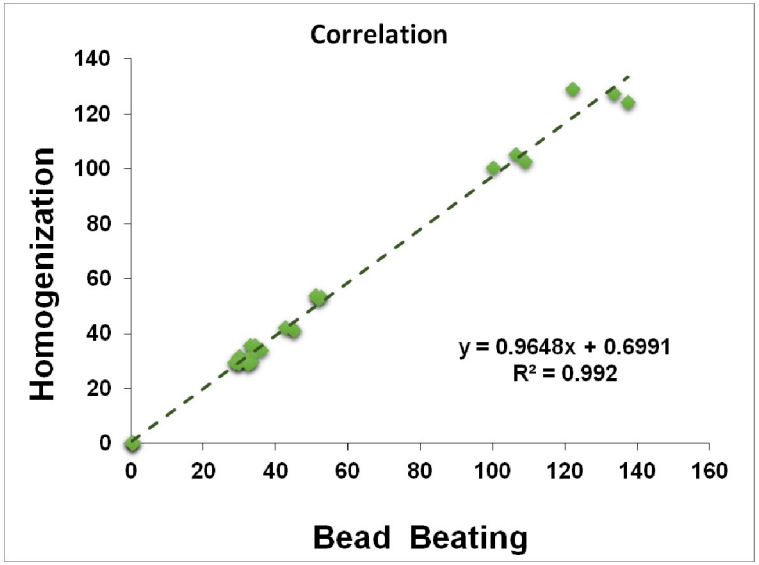
Comparative analysis

- We analyzed 9 samples (in triplicates) using both extraction methods
- Strong correlation of 99.2% between the methods.
- Paired t-Test suggests there's no significant difference between the two methods (p value > 0.05)



t-Test: Paired Two Sample for Means

	Homogenize	*Bead Beating*
Mean	51.137	50.038
Variance	1558.253	1462.248
Observations	27.000	27.000
Pearson Correlation	0.996	
Hypothesized Mean Difference	0.000	
df	26.000	
t Stat	1.549	
P(T<=t) one-tail	0.067	
t Critical one-tail	1.706	
P(T<=t) two-tail	**0.133**	
t Critical two-tail	2.056	

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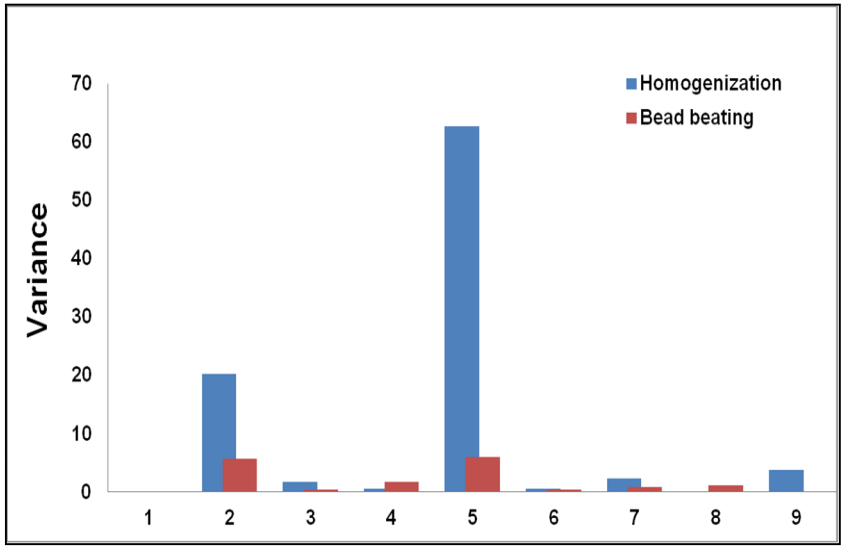
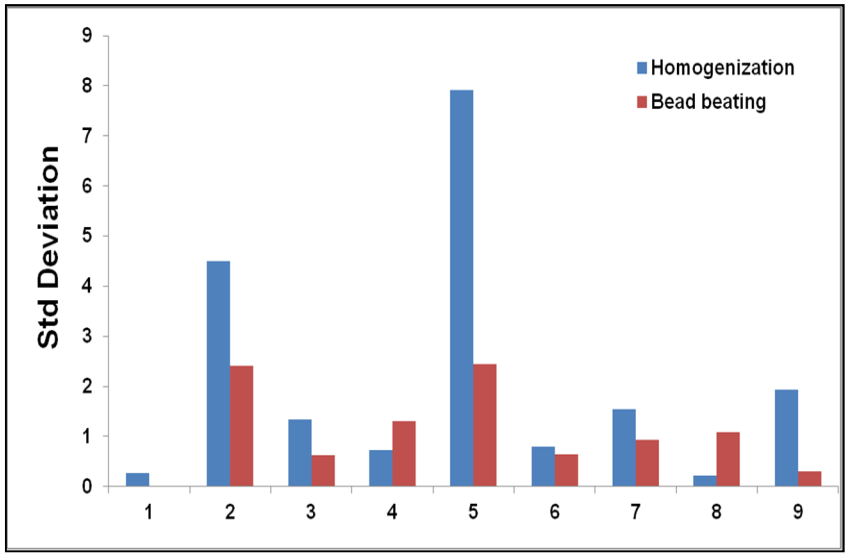
Comparative analysis

- The variance and the standard deviations by bead beating were mostly lower compared to that of the homogenization.





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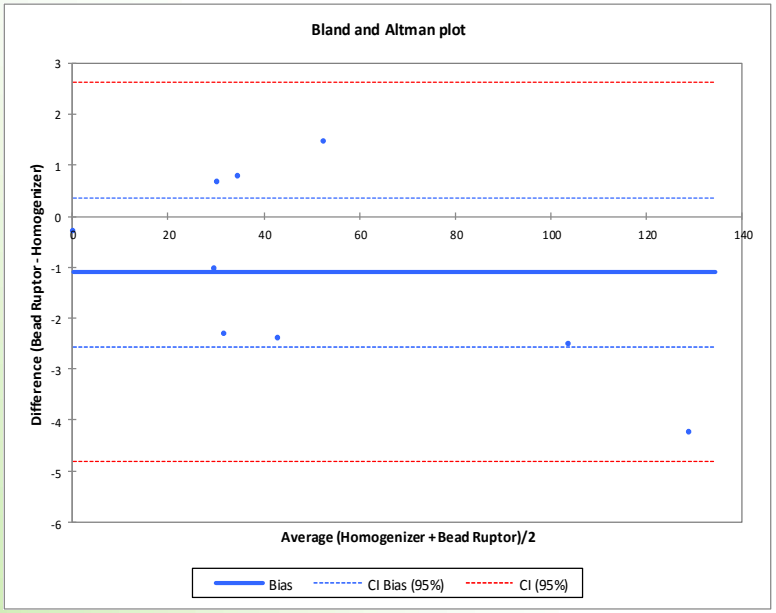
Method comparison using Bland-Altman method



Bland-Altman analysis:	
Bias	-1.096
Standard error	1.899
CI Bias (95%):	] -2.556 ,0.363[
CI (Differences):	] -4.818, 2.626[

t-test for two paired samples	
95% CI on the difference between means:	]-2.556,0.363[
Difference	-1.096
t (Observed value)	-1.732
t (Critical value)	2.306
DF	8
p-value (Two-tailed)	0.122
alpha	0.05

- The results indicate that the both the methods are comparable and there is no bias towards either method.

- The repeatability was observed to be better with bead beating. The p-value of 0.122 i.e. > alpha=0.05 and therefore the old method can be replaced by the new rapid method.

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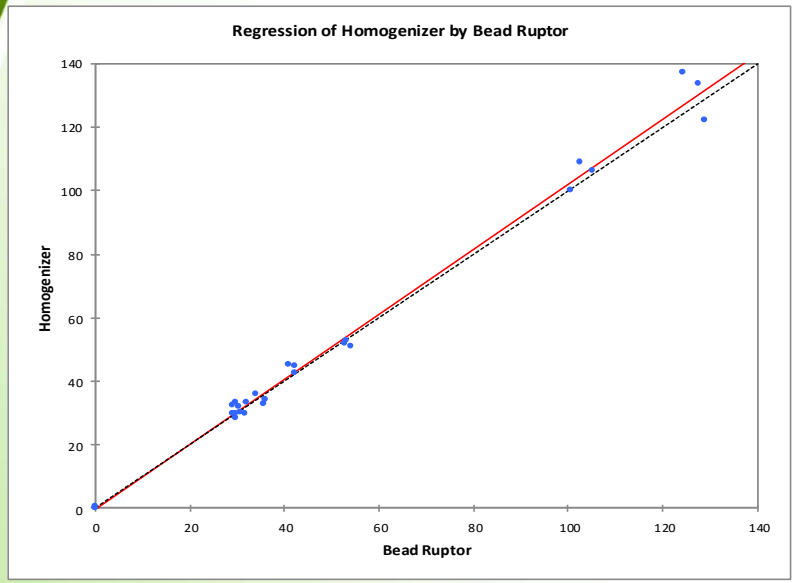
Method comparison by Passing and Bablok regression method:



Model coefficients:			
	Value	Lower bound 95% (Mean)	Upper bound 95% (Mean)
Intercept	-0.007	-1.612	1.281
Slope coefficient	1.020	0.976	1.064

Test of linearity / (Two-tailed test):	
Max(|Cusum|) (Observed value)	3.000
Max(|Cusum|) (Critical value)	6.925
p-value (Two-tailed)	0.879
alpha	0.05

- The results indicate that there is no systematic and no proportional difference between the two methods.

- Since the p-value 0.879 is greater than the significance level alpha=0.05, we conclude that there is no significant difference between the methods and one can be replaced by another.

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Conclusion

- The bead beating method of extracting chlorophyll a is much more efficient than the manual or semi-automatic method.

- The extraction process of the method is the most time consuming. By replacing the manual or semi-automatic mastication with bead beating, the number of samples that can be processed is increased **20 fold** with little or no loss of sample.

Summary

- Chlorophyll A levels
  - Give us a snapshot of the algal biomass in the water
  - This correlates with the nutrient load
- Chlorophyll A is only half of the story
  - The types of algae can tell us if our system is potentially toxic
  - If there is too much P or N
- Not all strains of cyanobacteria are toxic