



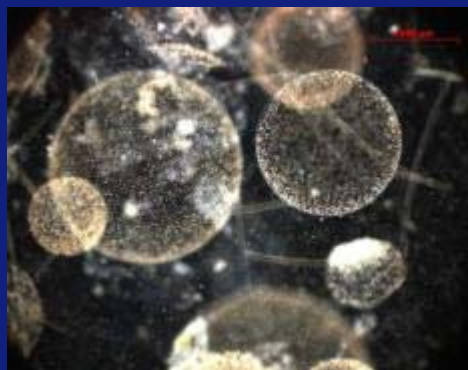
Royal Netherlands Institute for Sea Research



NORTH SEA BALLAST WATER

Lesson learnt from sampling and analysis during the Cetification: the NIOZ experience

Marcel Veldhuis
On behalf of the Ballast Water team



NIOZ is part of the Netherlands Organisation for Scientific Research (NWO)

Outline

- facility organization and funding
- facility location and physical/biological conditions
- overview of basic testing approaches and methods
- sampling and analysis
 - theoretical approach
 - practical approach
 - pragmatic approach
- Recommendations for compliance testing



Introduction NIOZ

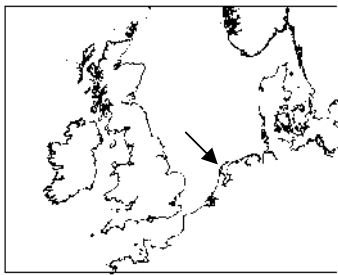
- NIOZ: fundamental research ca. 60-75 scientists
 - Census of Marine Life *CENSUS OF MARINE LIFE* 
 - Global network to assess and explain diversity of oceanic life
- EU-Marine Strategy framework Directive (2020)
 - EU's 6th Environment Action Programme (6EAP)
 - Biodiversity and bioinvasions
- EU-Interreg NS (50% funding; NIOZ & industry)
 - Ballast Water Opportunity project
 - Invasive organisms (mitigation)
 - Harmonization, control and research



NORTH SEA BALLAST WATER



facility location







Facility physical/biological conditions

- Wadden Sea; salinity 20 – 34 PSU;
 - temp: 0 – 20 °C,
 - TSS 5 – 400 mg/L NIOZ
 - POC 5 -20 mg/L
 - DOC 1 - 5 mg/L
 - >50 micron: $10^4 - 10^8 /m^3$
 - 10 – 50 micron 100 – $10^5 /ml$
 - bacteria $10^4 - 10^7 /mL$
 - mucus forming algae *Phaeocystis*



basic testing approaches and methods

- methods described in (scientific) literature
- well established for routine measurements (TSS, POC, DOC, salinity, pH, DO)
- special samples by ISO certified labs (human pathogens, ecotox tests) IMARES-Wageningen/C-mark
- In house developed (peer reviewed) methods addressing cell counts and viability
- Experimental methods (holistic approach), ongoing activity

basic testing approaches and methods

- Improve and expand present set of (multiple) test protocols (active substances)
- Compare present and future standard(s) with current achievements
- legal/statistical aspects of numbers and sample volumes
- (semi)automated analysis
 - TSS,POC,DOC, turbidity, salinity ~ 400 samples
 - Life -microscopy ($> 10 \mu\text{m}$) ~ 120 samples
 - Phytoplankton (PAM, FCM, micro) ~ 500 samples
 - Bacteria (counts, hum. Path.) ~ 500 samples
 - Viruses ~ 250 samples
 - Total ~ **1770 samples**

Experiences NIOZ

- Main guidance G8-guidelines (must strictly obeyed)
 - critical review of D2-Standard and guidelines
 - academic approach (detection of rare events, viability of various organisms)
- Relationship between organism number and sample size and counting errors
- More complex when dealing with natural community than with surrogate organisms

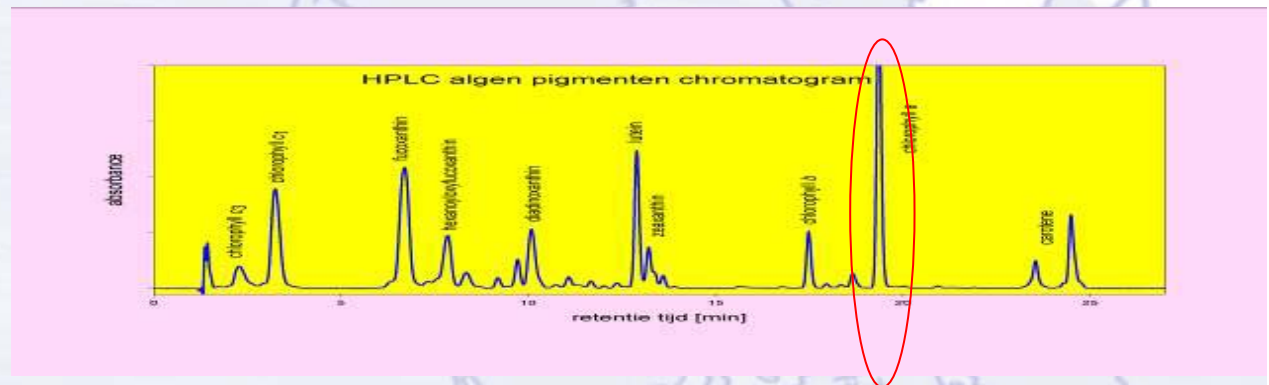
Experiences NIOZ

- Main guidance G8-guidelines (must strictly obeyed)
 - sample volume and replicates based on
 - 3 replicates of 1 m³ for >50 micron
 - 3 replicates of 1 ml for < 50 micron



Biology: the holistic approach

- Phytoplankton
- Biomass chlorophyll concentration; spectrofluorometric and HPLC (assessory pigments)

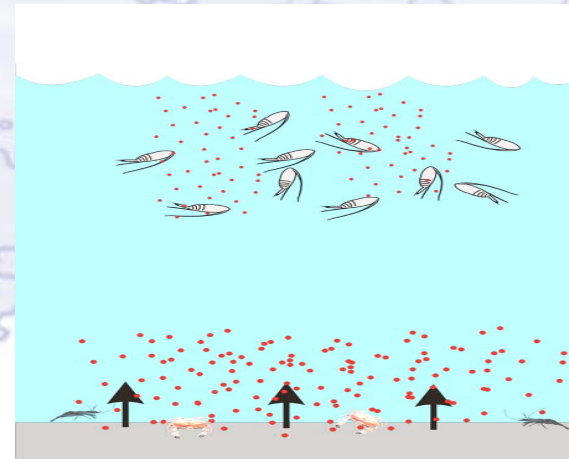
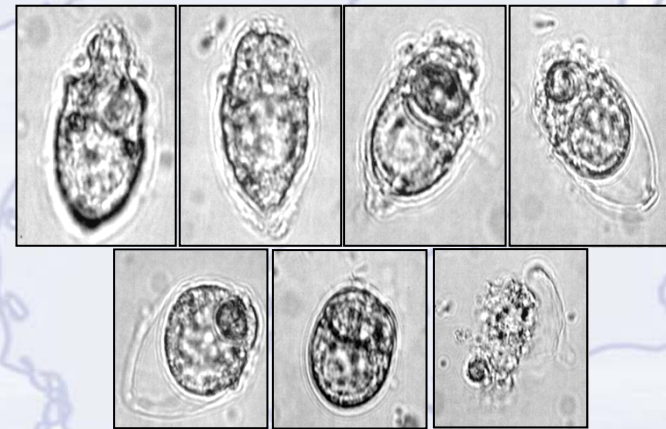


- Numerical abundance (microcopy, Flow cytometry, FlowCam)
- Viability PAM-fluorometry, cell-membrane (SYTOX Green) and re-growth incubations



Biology: the holistic approach

- Zooplankton
- Numerical abundance (microcopy, FlowCam)
- Viability (disrupted) body structure, movement
neutral red
- Crustaceae enzyme test (chitobiase, moulting enzyme)



Sampling and analysis (theory)

Sample size: E. Meshabi (BWO meeting Malmö 2010)

The formula

$$n = \frac{N z_{\alpha/2}^2 P(1-P)}{(N-1)e^2 + z_{\alpha/2}^2 P(1-P)}$$

- n = Sample size or the number of 1m³ ballast water samples that needs to be taken in order to have a true representation of the ballast water discharged.
- N = "population" size or total amount of ballast water discharged, in m³.
- P = probability of success or the estimated proportion of an attribute that is present in the "population". In this context it refers to the probability of having ≥10 viable organisms/m³ of ballast water sampled.
- $(1-P)$ = is the probability of failure (Q) that represents the probability of having < 10 viable organisms/m³ of ballast water sampled.
- $Z_{\alpha/2}$ = confidence coefficient for a given confidence interval (for a confidence level of 95%, $Z_{\alpha/2}=1.96$)
- e = standard sampling error: error that is assumed while the sampling is carried out. A value of 1-5% is considered as normal.

Sampling and analysis (theory)

Sample size: E. Meshabi (BWO meeting Malmö 2010)

Number of Samples

Sample sizes for the “populations” considered in the hypotheses and for a sampling error of 1%

“Population” of ballast water (m ³), N	3	8	24	216	1,000
Confidence Level	95%	95%	95%	95%	95%
Probability of success, P	0.5	0.5	0.5	0.5	0.5
Probability of failure, $(1-P)$	0.5	0.5	0.5	0.5	0.5
% of “Population” of ballast water required for sampling	100%	100%	100%	97.7%	90.6%
Number of 1 m ³ samples (m ³), n	3.00	7.9	23.9	211.2	905.7

Sampling and analysis the practical approach

Experiences NIOZ manual versus automated

■ manual

high load of detritus/mineral particles
direct information

subjective

objective

time consuming

less time consuming

low number of samples/replicates higher number samples/replicates

data stored for post-analysis

simple data archiving (species/#/viabY/N)

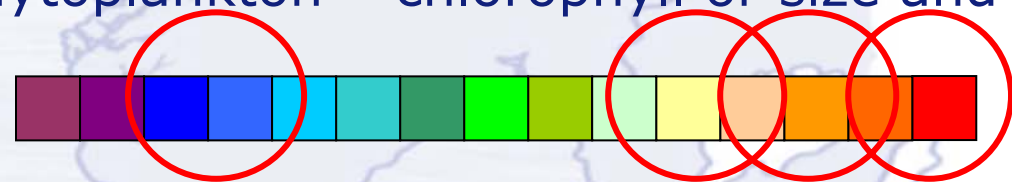
automated

expensive instruments



Numbers theoretical/practical considerations

- (automatic) detection of (rare) events
- Different selection criteria Phytoplankton – chlorophyll or size and the effect of sediment



flow cell

Fluorescence
and scatter
signals

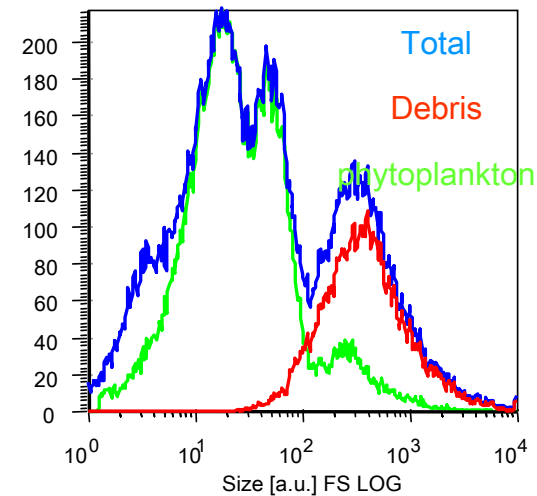
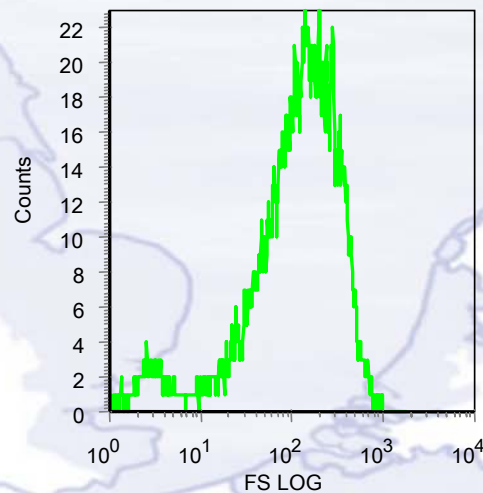
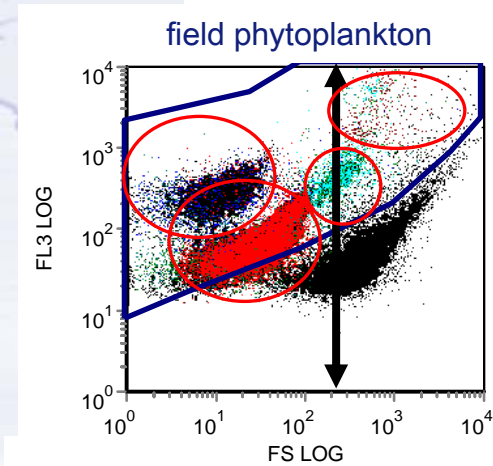
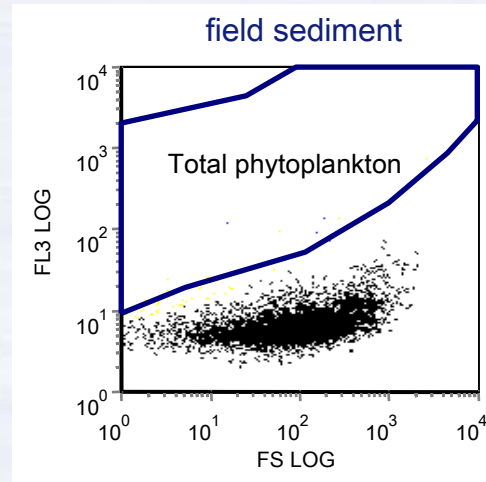
focused laser
beam

2 cm

- Flow cytometry
- Counting every single particle !!

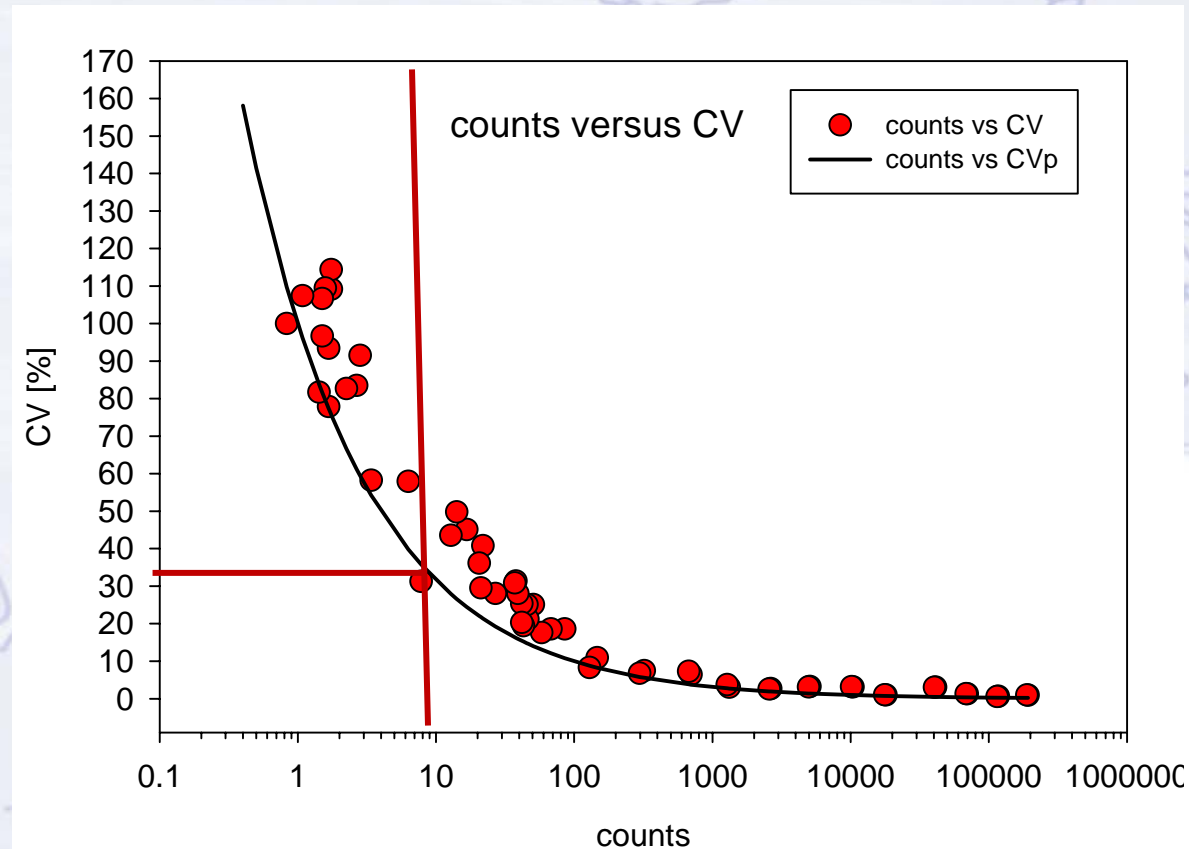
Numbers theoretical/practical considerations

- Discrimination between sediment and phytoplankton



Numbers theoretical considerations

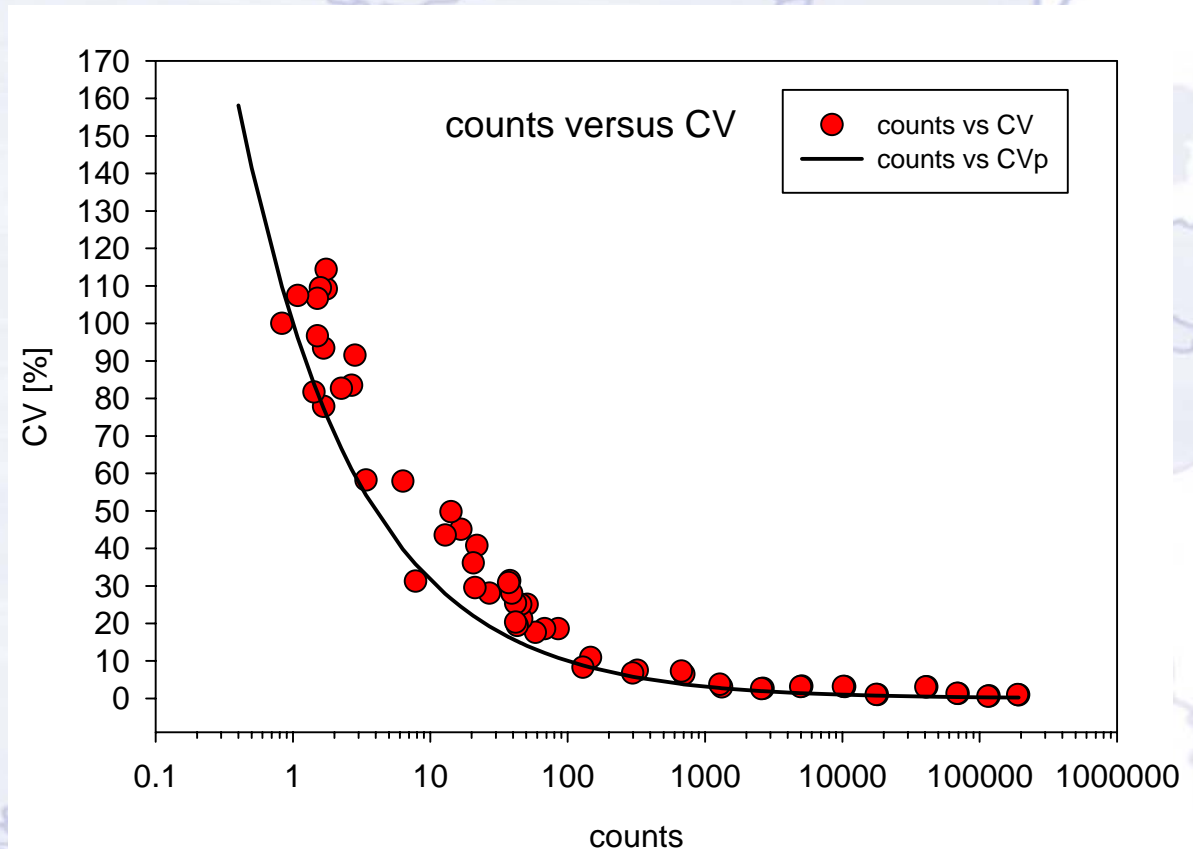
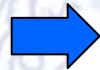
- Detection of (rare) events (Poisson's law)
- Coefficient of variance: $C_p = 100/\sqrt{n}$ (n=number of particles; independent of volume !!!)



Numbers; theoretical considerations

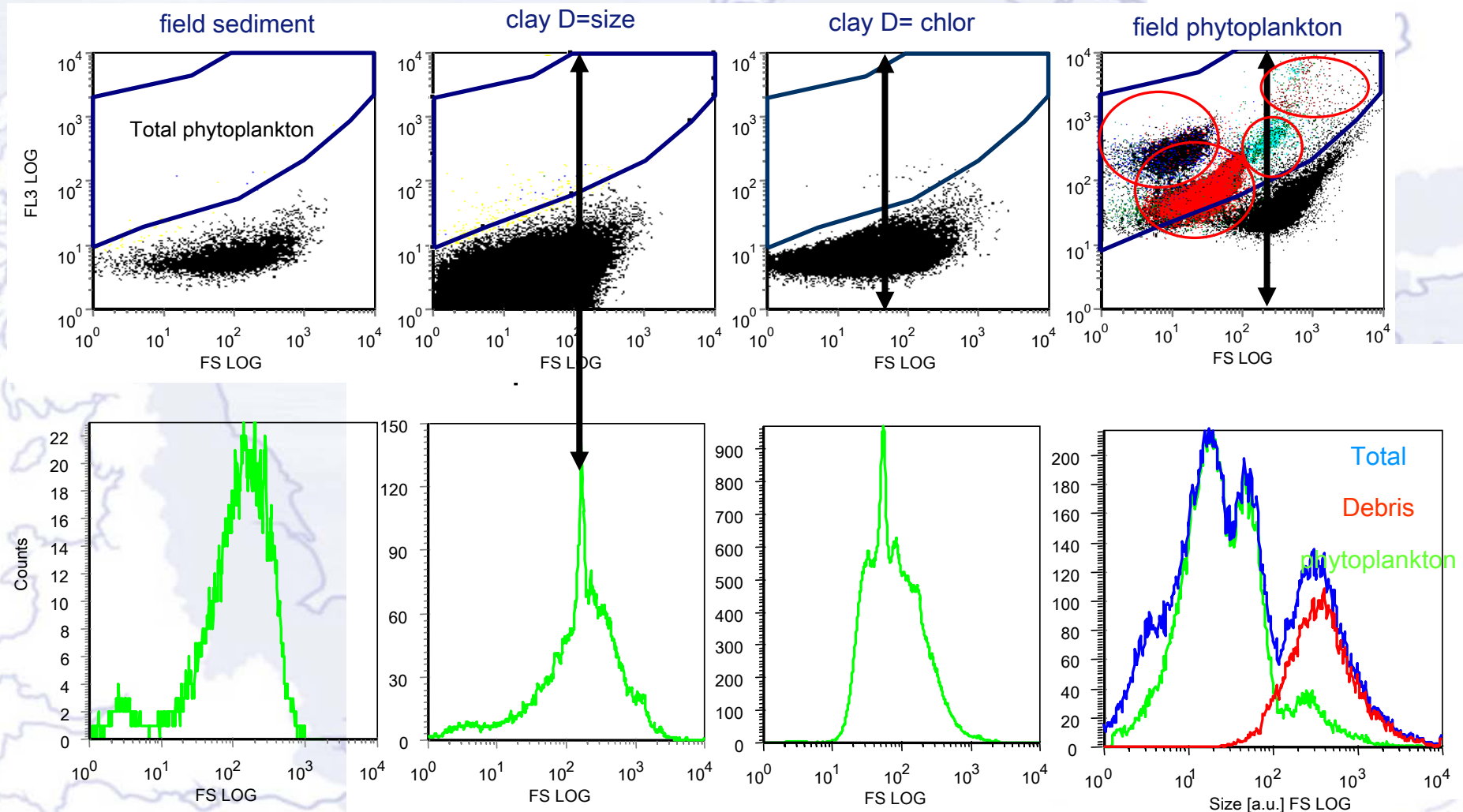
- Detection of (rare) events (Poisson's law)
- Coefficient of variance: $C_p = 100/\sqrt{n}$ (n=number of particles; independent of volume !!!)

number	CV %	min	max
1000000	0.10	999000	1001000
100000	0.32	99684	100316
10000	1.00	9900	10100
1000	3.16	968	1032
100	10.0	90	110
10	31.6	7.0	13.0
1	100.0	0.0	2.0
0.1	316.2	-0.2	0.4
0.01	1000	-0.09	0.11
0.001	3162	-0.031	0.033
0.0001	10000	-0.010	0.010
0.00001	31623	-0.003	0.0032
0.000001	100000	-0.0010	0.0010

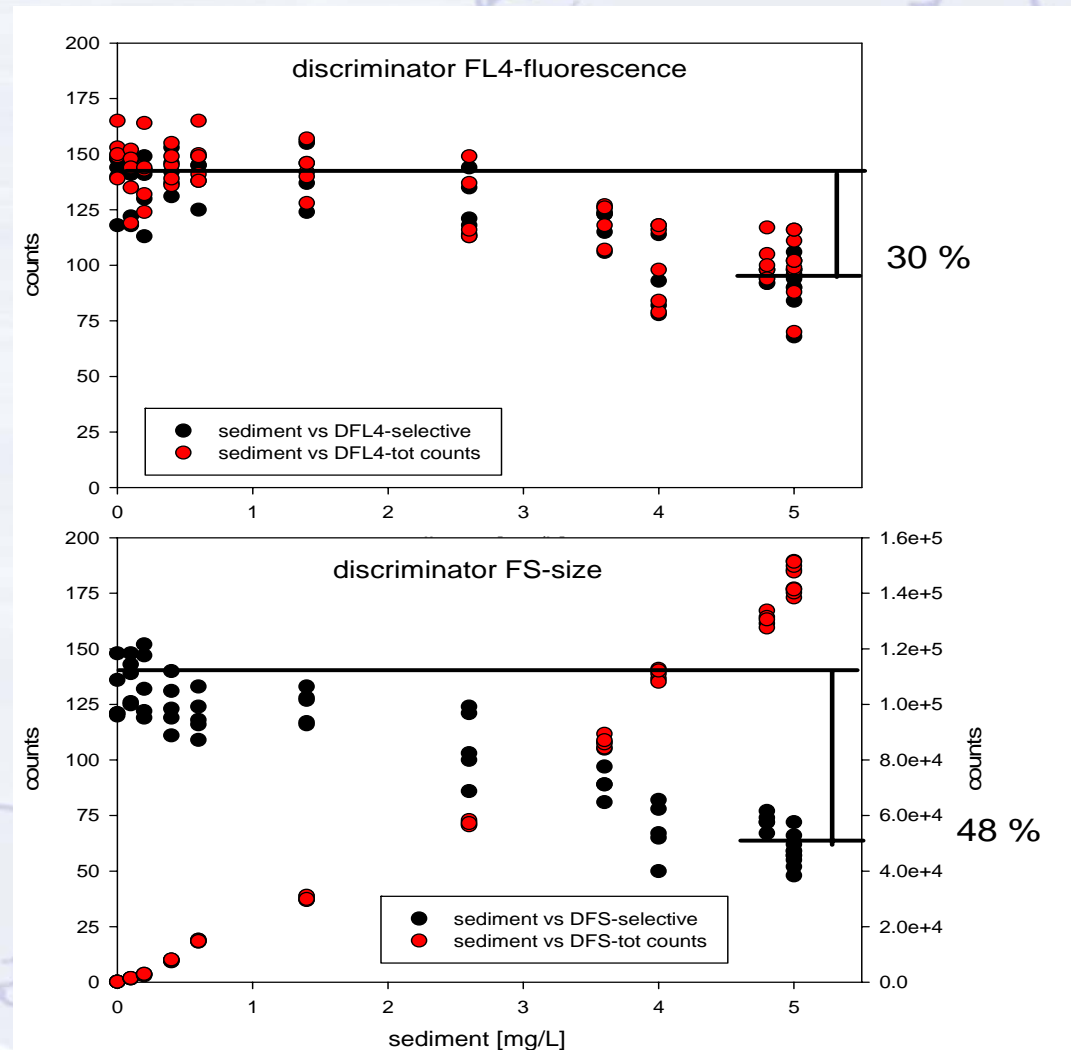


Numbers theoretical/practical considerations

- Different selection criteria phytoplankton – chlorophyll or size and the effect of sediment

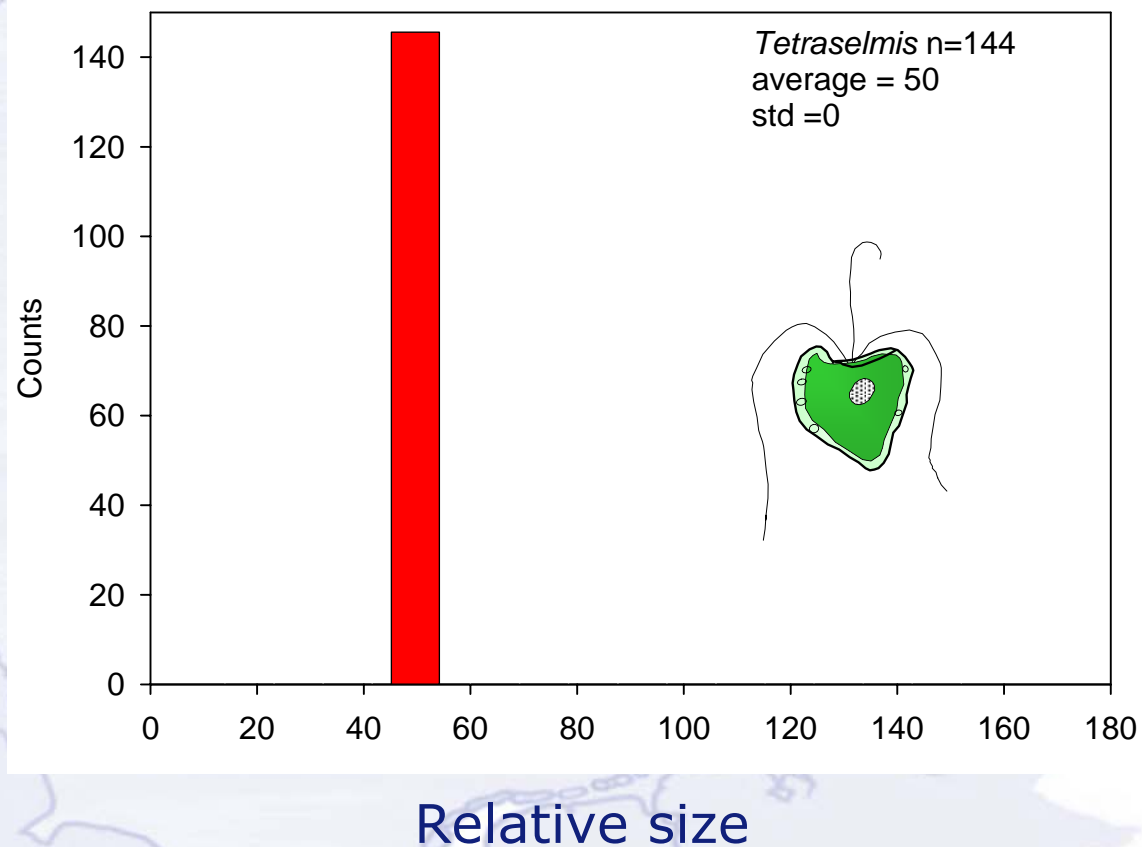


- Different selection criteria phytoplankton – chlorophyll or size and the effect of sediment
- counts as function of increasing amount of clay



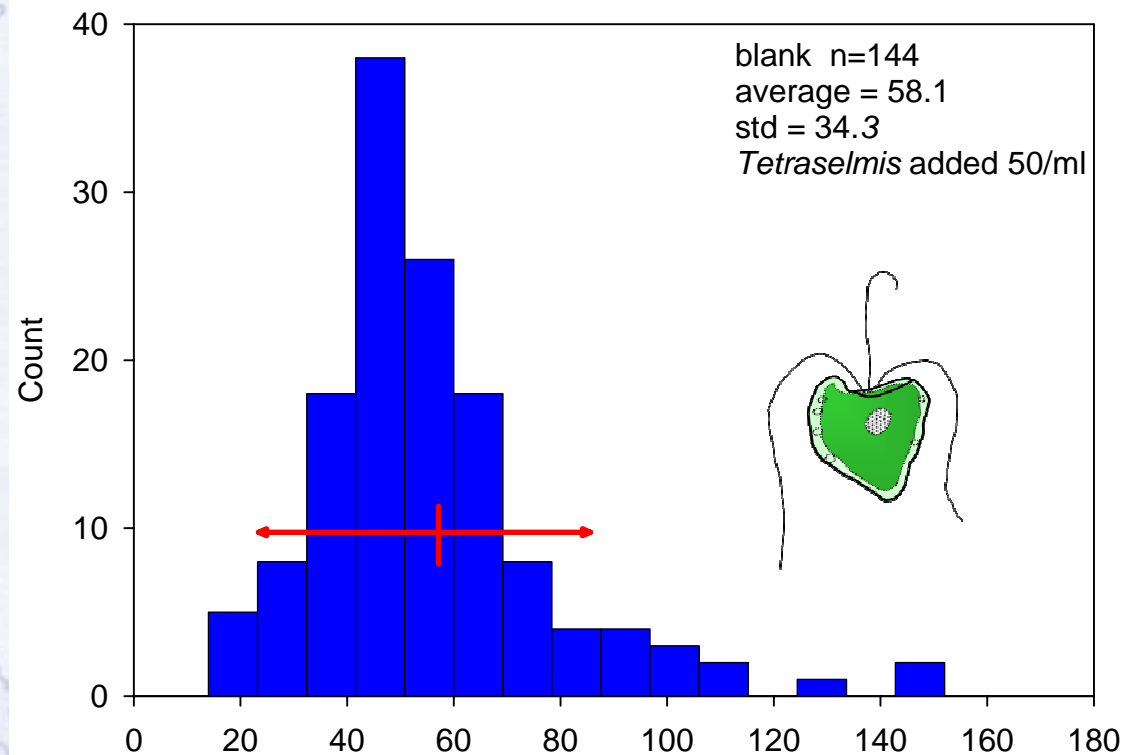
Counting organisms

What about size: the ideal organism

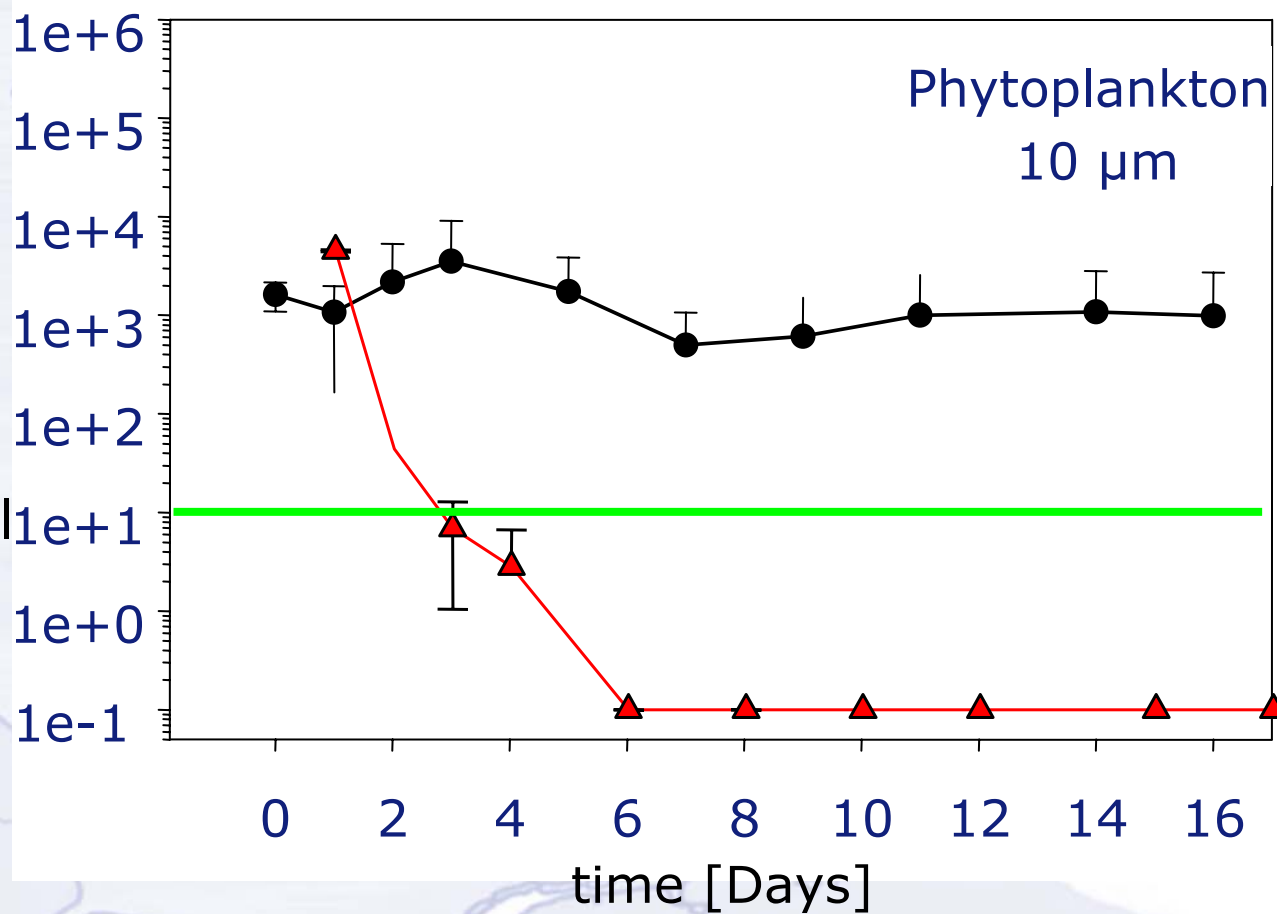
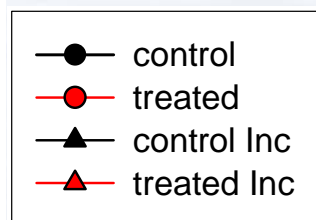


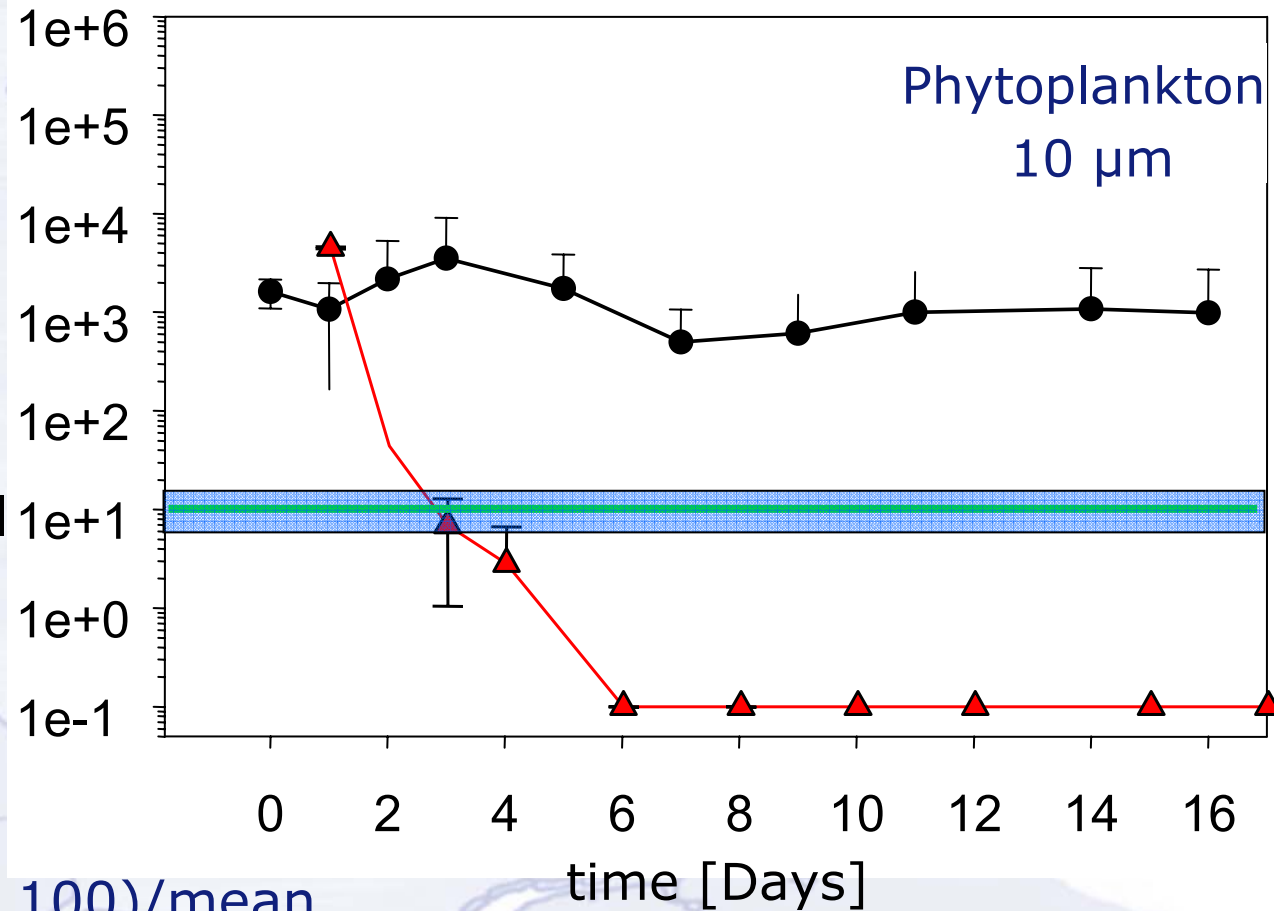
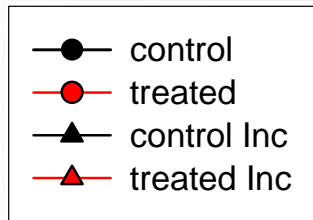
Counting organisms

What about size: the real world



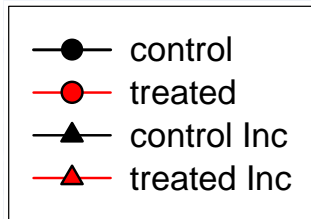
Relative size
normal distribution



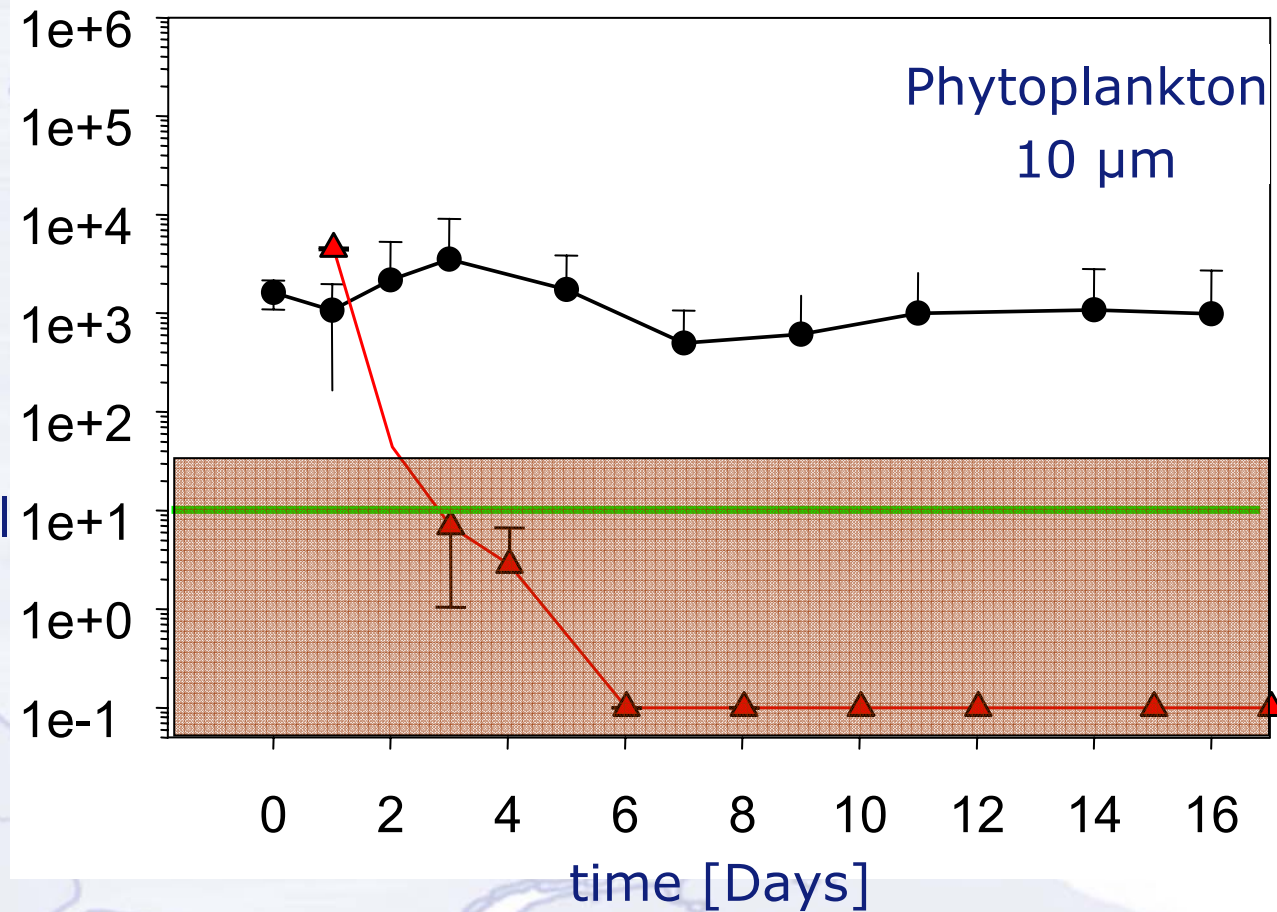


max 10/ml

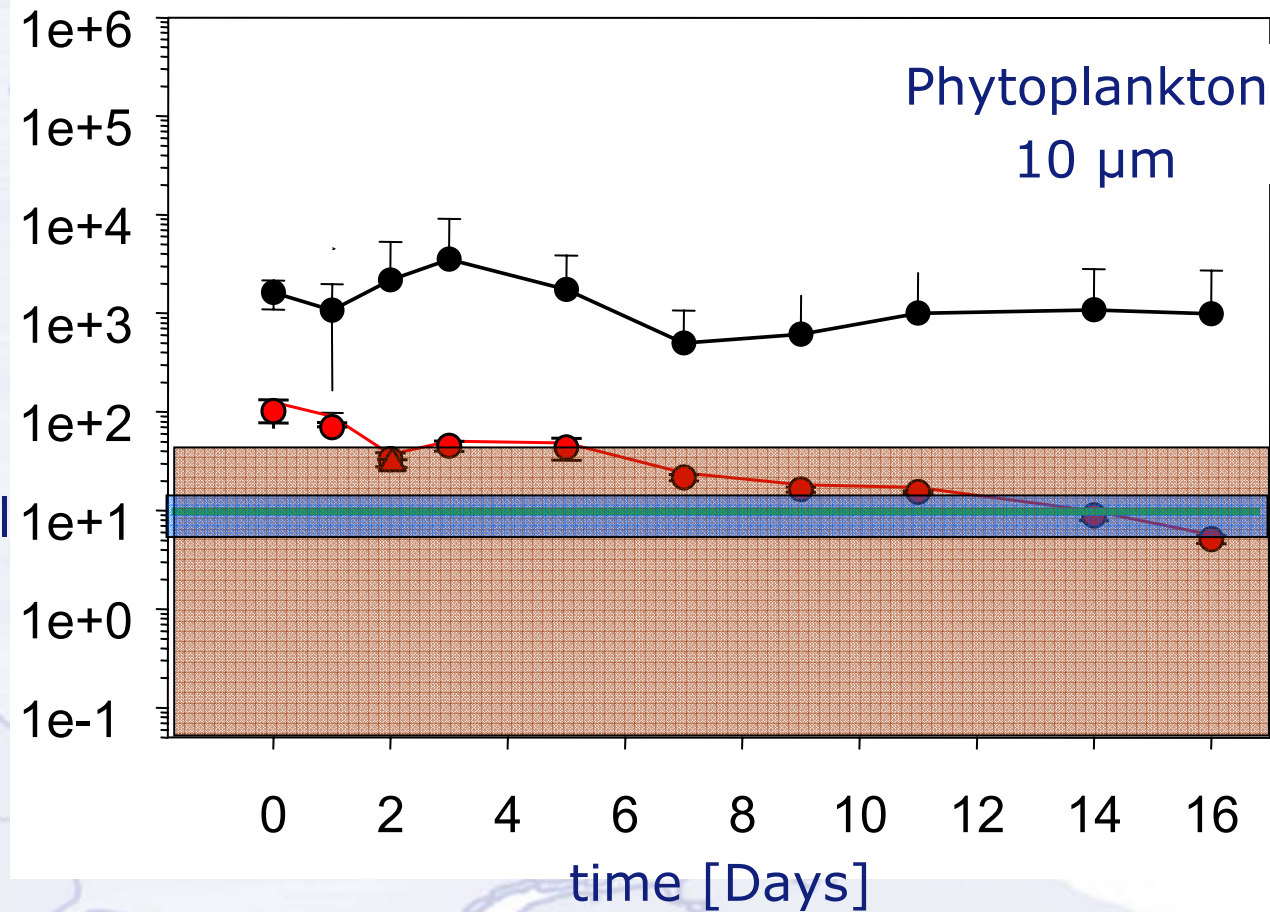
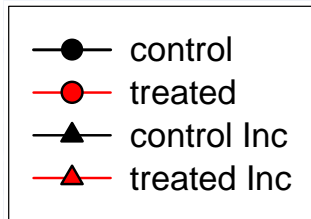
CV = (Std * 100)/mean
CV 30% and mean = 10
Std = 3
Range 7 – 13 !!!



max 10/ml



Std = 20 – 50 due to sediment particles

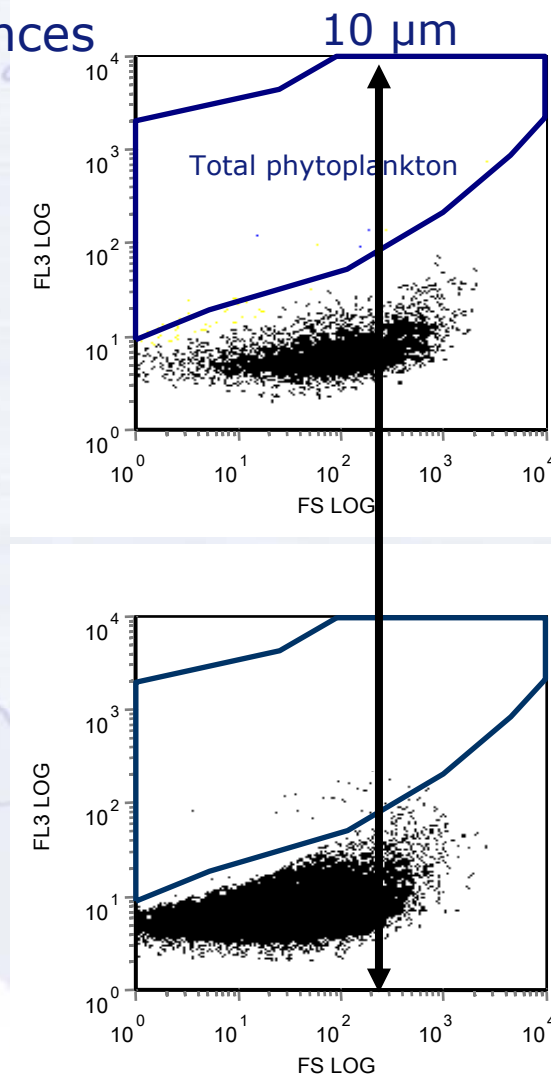


Std = 20 – 50 due to sediment particles

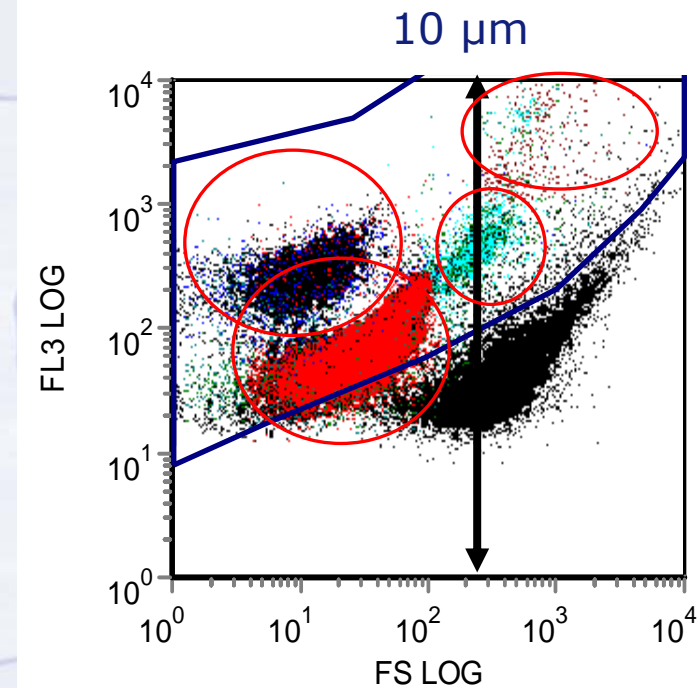
Differences between technologies

Active substances UV-radiation

Active substances



UV-radiation



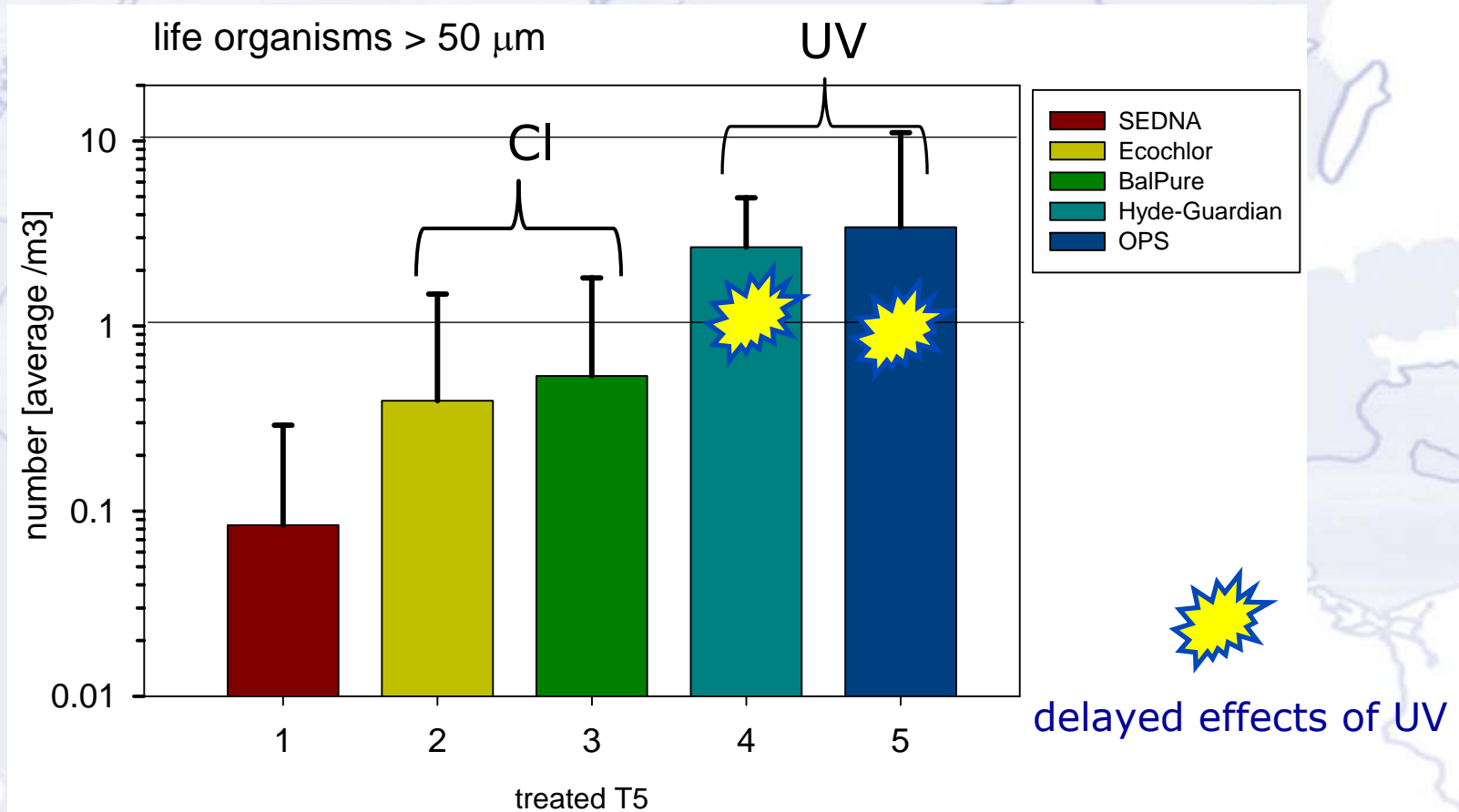
Additional data like

- photosynthetic efficiency
- membrane integrity

Reality: legally defensible data

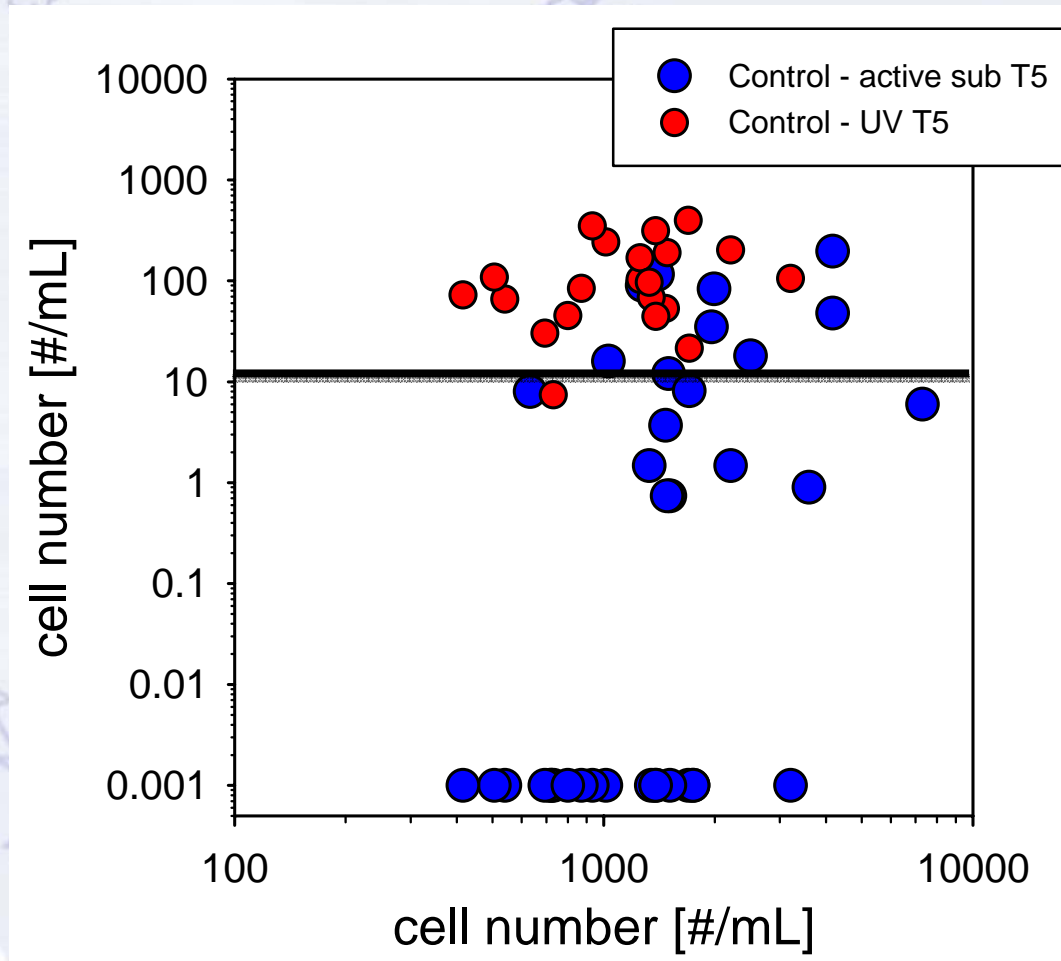
Current status BWT technologies

benchmark: $> 50 \mu\text{m}$ organisms



!!! On average 1 order of magnitude lower than D2-Standard !!!

Reality: legally defensible data
Current status BWT technologies
benchmark: phytoplankton 10 -50 micron



!!! Not always clear, multiple methods required !!!

Pragmatic approach

- aspects to be considered (practical constraints)
 - Dead volume, piping and tanks (technical solution)
 - Average sample, representative volume/time of deballasting
- fixed volume and fixed number of replicates
- fixed volume analyzed (exact 1 ml, concentration steps)
- (semi) automated analysis, data archiving

- potential regrowth of organisms in ballast water tank over time



org. number counted	1	1	1	1	1	1	1
sample volume	10 L	100 L	per m3	10 m3	100 m3	1000 m3	10000 m3
number/m3	100	10	1	0.1	0.01	0.001	0.0001
Standard m3	10	100	10	1	0.1	0.01	0.001
	1	100	10	1	0.1	0.01	0.001
	0.1	100	10	1	0.1	0.01	0.001
	0.01	100	10	1	0.1	0.01	0.001
	0.001	100	10	1	0.1	0.01	0.001
3 replicates							
org. number counted	1	1	1	1	1	1	1
sample volume	10 L	100 L	per m3	10 m3	100 m3	1000 m3	10000 m3
number/m3	33.3	3.3	0.33	0.03	0.003333	0.000333	3.33E-05
Standard m3	10	33.3	3.3	0.33	0.03	0.003333	0.000333
	1	33.3	3.3	0.33	0.03	0.003333	0.000333
	0.1	33.3	3.3	0.33	0.03	0.003333	0.000333
	0.01	33.3	3.3	0.33	0.03	0.003333	0.000333
	0.001	33.3	3.3	0.33	0.03	0.003333	0.000333
org. number counted	10	10	10	10	10	10	10
sample volume	10 L	100 L	per m3	10 m3	100 m3	1000 m3	10000 m3
number/m3	1000	100	10	1	0.1	0.01	0.001
Standard m3	10	1000	100	10	1	0.1	0.01
	1	1000	100	10	1	0.1	0.01
	0.1	1000	100	10	1	0.1	0.01
	0.01	1000	100	10	1	0.1	0.01
	0.001	1000	100	10	1	0.1	0.01

Cracking the brains

Large organisms
> 50 micron



D2-Standard

assuming BWT system with efficiency of 10 organism/m3			
	# samples		
sample volume	to count 1	numbers per m3	
10 L	10	100	
100 L	1	10	
1 m3	0.1	1	
10 m3	0.01	0.1	

assuming BWT system with efficiency of 1 organism/m3			
	# samples		
sample volume	to count 1	numbers per m3	
10 L	100	100	
100 L	10	10	
1 m3	1	1	
10 m3	0.1	0.1	

upsampling: not possible to count 0.1 viable organism			
assuming BWT system with efficiency of 0.1 organism/m3			
	# samples		1 org. /10 m3
sample volume	to count 1	numbers per m3	
10 L	1000	100	
100 L	100	10	
1 m3	10	1	
10 m3	1	0.1	

upsampling: not possible to count 0.01 viable organism			
assuming BWT system with efficiency of 0.01 organism/m3			
	# samples		1 org. /100 m3
sample volume	to count 1	numbers per m3	
10 L	10000	100	
100 L	1000	10	
1 m3	100	1	
10 m3	10	0.1	

Cracking the
brains

Large
organisms
> 50 micron

Phase2 USCG

assuming BWT system with efficiency of 10 organism/m³

	# samples			
sample volume	to count 1	numbers per m ³		
10 L	10	100		10
100 L	1	10		
1 m ³	0.1	1		
10 m ³	0.01	0.1		

visits for non-compliance

Cracking the brains

assuming BWT system with efficiency of 1 organism/m³

	# samples			
sample volume	to count 1	numbers per m ³		
10 L	100	100		100
100 L	10	10		10
1 m ³	1	1		
10 m ³	0.1	0.1		

Large organisms
> 50 micron

upsampling: not possible to count 0.1 viable organism
assuming BWT system with efficiency of 0.1 organism/m³

	# samples		1 org. /10 m ³	
sample volume	to count 1	numbers per m ³		
10 L	1000	100		1000
100 L	100	10		100
1 m ³	10	1		10
10 m ³	1	0.1		

upsampling: not possible to count 0.01 viable organism
assuming BWT system with efficiency of 0.01 organism/m³

	# samples		1 org. /100 m ³	
sample volume	to count 1	numbers per m ³		
10 L	10000	100		10000
100 L	1000	10		1000
1 m ³	100	1		100
10 m ³	10	0.1		10

assuming BWT system with efficiency of 10 organism/mL					
	# samples				
sample volun	to count 1 numbers per mL			visits for non-compliance	
1 mL	0.1	1			
10 mL	0.01	1			
100 mL	0.001	1			
1 L	0.0001	1			
assuming BWT system with efficiency of 1 organism/mL					
	# samples				
sample volun	to count 1 numbers per mL				
1 mL	1	1			
10 mL	0.1	1			
100 mL	0.01	1			
1 L	0.001	1			
upscaling: not possible to count 0.1 viable organism					
assuming BWT system with efficiency of 0.1 organism/mL					
	# samples		1 org. /10 mL		
sample volun	to count 1 numbers per mL				
1 mL	10	1		10	
10 mL	1	1		1	
100 mL	0.1	1			
1 L	0.01	1			
upscaling: not possible to count 0.01 viable organism					
assuming BWT system with efficiency of 0.01 organism/mL					
	# samples		1 org. /100 mL		
sample volun	to count 1 numbers per mL				
1 mL	100	1		100	
10 mL	10	1		10	
100 mL	1	1		1	
1 L	0.1	1			

Cracking the brains

small organisms
< 50 micron

challenges for Compliance

- numerous
- based on practical and pragmatic considerations
- straightforward sampling procedure; preferably based on fixed sample volumes/sampling times etc

Thank you for your attention



