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In vivo chlorophyll fluorescence measurements

Comparing the YSI 6025 sensor with the standardized NEN 6520 analyses

Deltares

Title
In vivo chlorophyll fluorescence measurements

Client	Project	Reference	Pages
Rijkswaterstaat Waterdienst	1205981-002	1205981-002-VEB-0001-vj	19

Key words
Chlorophyll, sensors, probes, spectrophotometry, algae, temperature.

Summary
Phytoplankton biomass is often described by the concentration of the chlorophyll *a* (Chl *a*) pigment. Chl *a* also fluoresces and therefore chlorophyll concentration can be quantified in vitro (i.e. spectrophotometrically) by its absorption coefficient and then via a calibrated fluorometer, by its fluorescence intensity. In situ chlorophyll sensors (fluorometers) directly measure the fluorescence of chlorophyll in living cells which makes them candidates for real-time data collection. Although fluorometers are an easy method for collecting large quantities of data, there are variables associated with in situ fluorescence that result in errors and interference. The fluorescence for a given cell concentration is affected by a number of factors including the amount of light the cell was exposed to prior to the measurement and variation among different species, physiological states and environmental conditions.

Rijkswaterstaat wants to implement in situ measuring techniques for chlorophyll as an alternative to the more classical chlorophyll determination (spectrophotometric method). However, these in situ measuring techniques for chlorophyll, fluorometers, do not measure chlorophyll as exactly as in the lab. The aim of this study is a comparison between an in situ chlorophyll sensor (YSI 6025) and the spectrophotometric method for chlorophyll (NEN6520:2006) and to find explanations for possible deviations. The research question is how much chlorophyll measurements, as determined with a fluorometer, deviate from spectrophotometric chlorophyll analyses? Sub questions are: what are the upper and lower limits of the YSI 6025 chlorophyll sensor and which factors influence possible differences between YSI and NEN measurements? To answer these questions, in vivo fluorescence from 2011 monitoring among several locations was compared to NEN measurements and other environmental variables.

Chlorophyll fluorescence as measured by the YSI 6025 chlorophyll sensor correlated well with spectrophotometric measurements (r=0.85). About 72% of the chlorophyll fluorescence was explained by in vitro (i.e. spectrophotometric analyses on chlorophyll) variation. The remaining variation in fluorescence could, however, not be explained by variation in other measured environmental variables (e.g. temperature, suspended solids concentration, transparency). Most variation was found at low chlorophyll concentrations (< 5.2 µg Chl/L).

The median lower limit of the YSI 6025 chlorophyll sensor at which it can be compared to the NEN-method was 5 µg Chl L^{-1}. This is higher than the spectrophotometric readings, -probably as a result of detection of free algal pigments by the YSI sensor. Deltares advices to compare NEN and YSI using a serial dilution. This should be done preferably with water from the location of interest if information of the algal composition is known. Otherwise laboratory cultured algal strains should be used. The upper limit of the YSI 6025 chlorophyll sensor could not be established with the current data set. At high chlorophyll peaks, the YSI sensor always resulted in lower chlorophyll concentration estimates than the NEN analyses. This is probably the result of shading effects and lower fluorescence yield per Chl *a* under low light conditions.

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The YSI 6025 sensor seems a promising tool which can be used complementary to standardized spectrophotometric analyses. To find out what the unexplained variation is between the YSI and NEN, Deltares advices to include CDOM and algal composition (and concentration) in the field monitoring program. The YSI 6025 chlorophyll sensor should be calibrated with the phytoplankton assemblage of the location of interest to obtain correct chlorophyll readings.

Versie	Datum	Auteur	Paraaf	Review	Paraaf	Goedkeuring	Paraaf
	Dec. 2012	Miguel Dionisio Pires		Bas van der Zaan		Lucas Janssen	

State
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Inhoud

1 Introduction

Knowledge of aquatic ecosystems starts with phytoplankton because of their role in structuring ecosystems. Phytoplankton biomass is often described by the concentration of the chlorophyll *a* (Chl *a*) pigment, which plays a fundamental role in photosynthesis. The Chl *a* molecule is optically interesting in that it has two distinct absorption peaks in the visible spectrum (Figure 1.1). This molecule also fluoresces and therefore chlorophyll concentration can be quantified in vitro (i.e. spectrophotometrically) by its absorption coefficient (UNESCO 1966) and then via a calibrated fluorometer, by its fluorescence intensity (Lorenzen 1966).

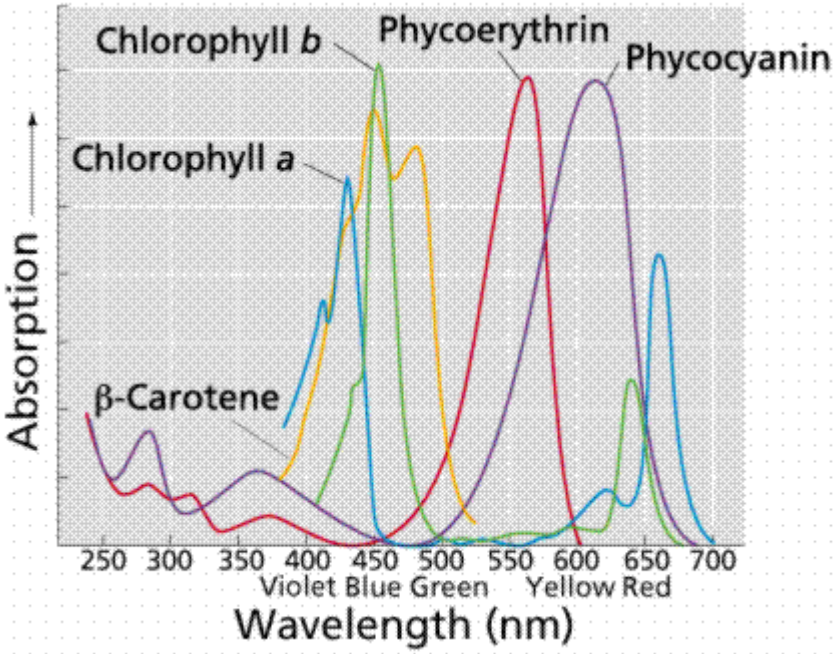

Figure 1.1 *Absorption spectra of different phytoplankton pigments. The pigment of interest in this study, Chl a, has two distinct peaks at 465 and 665 nm (blue line). Figure from Purves et al. (2004).*

In situ chlorophyll sensors (also called fluorometers) have been used in the past 40 years (Lorenzen 1966). They directly measure the fluorescence of chlorophyll in living cells and this makes them candidates for real-time data collection. These in situ sensors have certain benefits as ease of handling, speed and the ability to collect large quantities of data. Although fluorometers are an easy method for collecting large quantities of data, there are variables associated with in situ fluorescence that result in errors and interference. The fluorescence for a given cell concentration is affected by a number of factors including the amount of light the cell was exposed to prior to the measurement and variation among different species, physiological states and environmental conditions.

Rijkswaterstaat wants to implement in situ measuring techniques for chlorophyll as an alternative to the more classical chlorophyll determination (spectrophotometric method). However, these in situ measuring techniques for chlorophyll, fluorometers, do not measure chlorophyll as exactly as in the lab. The aim of this study is a comparison between an in situ chlorophyll sensor (YSI 6025) and the spectrophotometric method for chlorophyll *a* (NEN6520:2006) and to find explanations for possible deviations.

The research question is how much chlorophyll measurements, as determined with a fluorometer, deviate from spectrophotometric chlorophyll analyses? Sub questions are: what are the upper and lower limits of the YSI 6025 chlorophyll sensor and which factors influence possible differences between YSI and NEN measurements? Data obtained from the field (several locations) for different parameters in 2011 were used (See Methods) to answer these questions.

2 Methods

2.1 Sampling

In situ chlorophyll measurements in 2011 (whole year) were performed at 21 freshwater systems (all locations of Rijkswaterstaat) using the YSI 6025 chlorophyll sensor (as described in the manual of the manufacturer). Several other parameters, as temperature, pH, light extinction etc., were also recorded. For many locations also samples were taken for spectrophotometric chlorophyll measurements (NEN6520:2006). This NEN method is assumed to provide true chlorophyll data of the water samples. At 18 of the 21 locations in which the YSI 6025 sensor was applied, samples were taken for NEN chlorophyll determinations according to NEN.

The 18 locations used for data analyses were: Amerikahaven-2, Amsterdam (kilometer 25, IJtunnel), Broekerhaven, Den Oever, Eemmeerdijk (kilometer 23) Hoornsche Hop, Houtribhoek, Ketelmeer west, Marken Gouwzee, Markermeer midden, Pampus oost, Ramsdiep (kilometer 10), Steile bank, Veluwemeer midden, Vrouwezand, Westhaven-2, Zijkanaal D-1 and Zijkanaal E.

Measurement frequencies during 2011 ranged from 4 (Zijkanaal E) to 10 times (Eemmeerdijk, Ketelmeer west, Pampus oost, Veluwemeer midden).

2.2 Data analyses

YSI and NEN data for the 18 locations (and same sampling dates) were correlated to each other. The residuals from the regression of YSI against NEN data (i.e. eliminating the effect of NEN data) were then plotted against other parameters to see if the remaining variation in the YSI data could be attributed to other (independent) variables. YSI and NEN data were normalized by Box-Cox transformations. Statistics were performed with the statistical software package STATISTICA (version 10).

3 Results

Figure 3.1 shows the chlorophyll concentrations at the 18 locations as determined both with the YSI sensor and by spectrophotometry (NEN) from April 2011 until January 2012. At low chlorophyll concentrations both methods roughly follow the same pattern. Sometimes the YSI gives higher results than the NEN measurements and sometimes NEN measures higher concentrations. At high chlorophyll concentrations, however, NEN measurements always produce higher results then the YSI sensor (Figure 3.1).

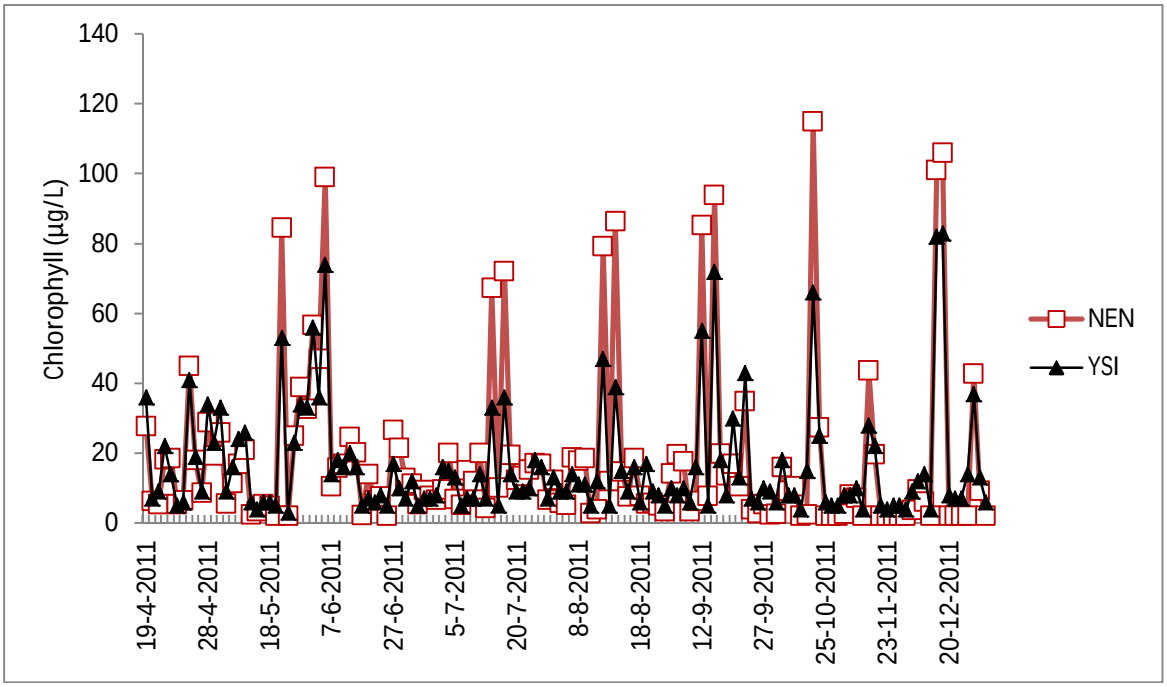

Figure 3.1 *Chlorophyll concentrations at 18 locations from April 2011 – January 2012 as determined with the YSI 6025 sensor (white rectangular with red line) and NEN (everything black). Every time a location was visited, a measurement with YSI 6025 took place and a sample for NEN was taken. Data are therefore single measurements in time and not averages. Note: the lines between data points do not imply a relationship between data points. They are simply placed in the figure to show which YSI data point is paired with which NEN data point.*

Chlorophyll concentrations measured with the YSI sensor correlated well with spectrophotometric analyses (NEN; Figure 3.2; r=0.85). The coefficient of variation is 0.72 which means that 28% of the variation in the YSI data cannot be explained by variation in NEN data. There are therefore other factors that may influence variation in the YSI fluorescence data. Figure 3.2 shows that the variation between YSI and NEN is higher at low than at high NEN values (below and above 2.7 µg Chl/L (Box-Cox transformed, which corresponds to an original NEN value of 23.3 µg Chl/L), respectively). The R^2 for NEN values < 2.7 µg Chl/L was 0.49, while the R^2 for NEN values > 2.7 µg Chl/L was 0.69. Most of the variation between the YSI 6025 sensor and the NEN analyses for chlorophyll seem therefore to exist at the lower chlorophyll concentrations (see also further below).

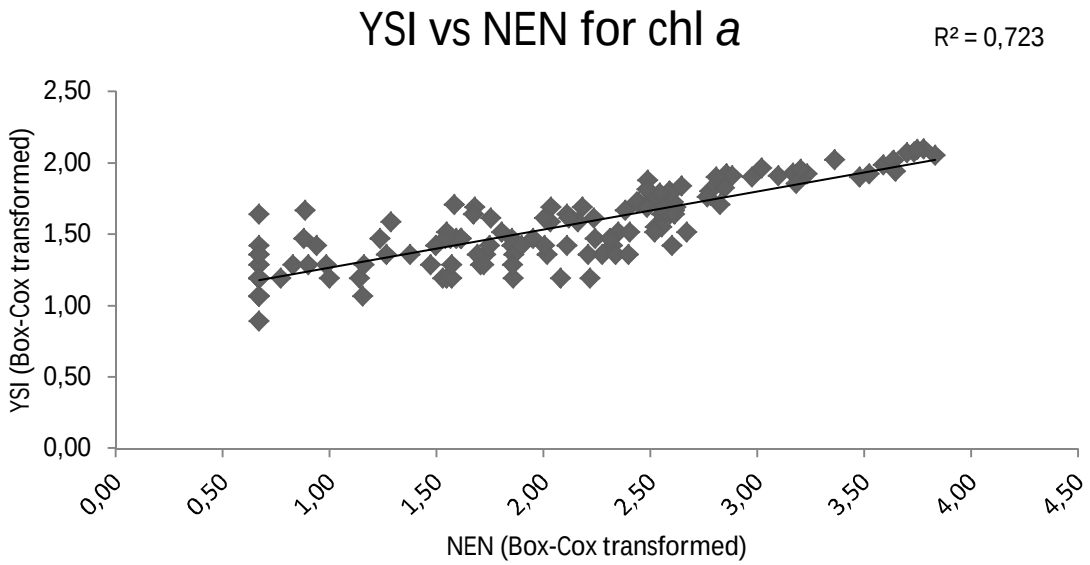

Figure 3.2 *Correlation between YSI 6025 and NEN chlorophyll measurements. Data were Box-Cox transformed to meet the assumptions of normality.*

To find out which other factors may influence variation in the YSI fluorescence data, residuals calculated from the regression in Figure 3.2 were plotted against factors that were measured at the same time as chlorophyll (i.e. both NEN and YSI) and that are known or suspected to influence fluorescence. These factors are: concentration of suspended solids, transparency, the extinction coefficient, water temperature, depth, degree of cloudiness, pH and locations. Results are shown in Figures 3.3-3.10.

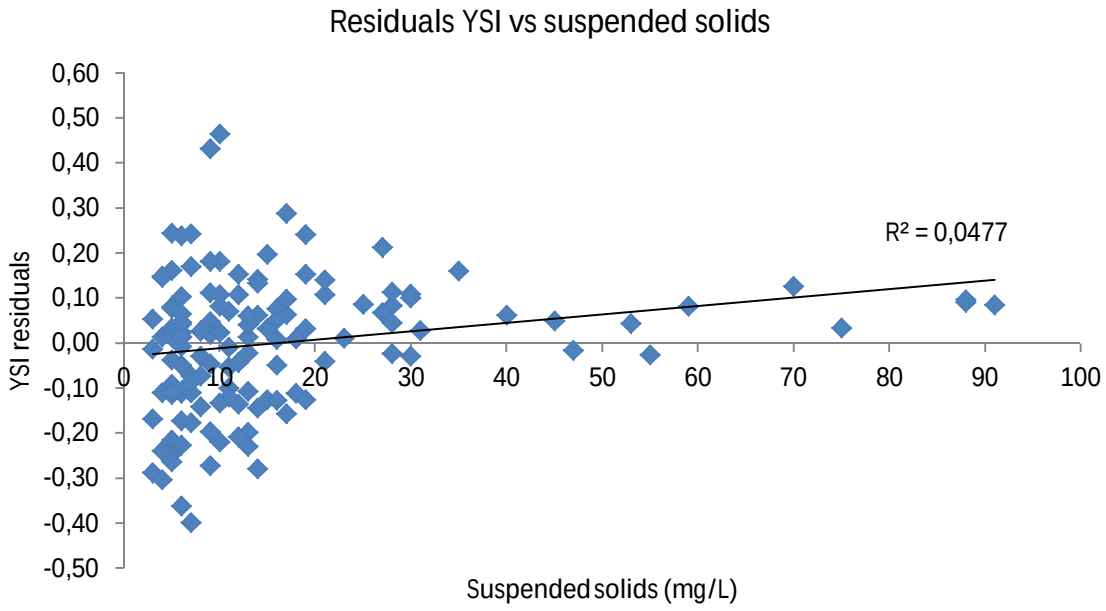

Figure 3.3 *Residuals (µg Chl/L) from the YSI fluorescence regression against NEN chlorophyll measurements (Figure 3.2) plotted against the concentration of suspended solids measured at the time of measurements.*

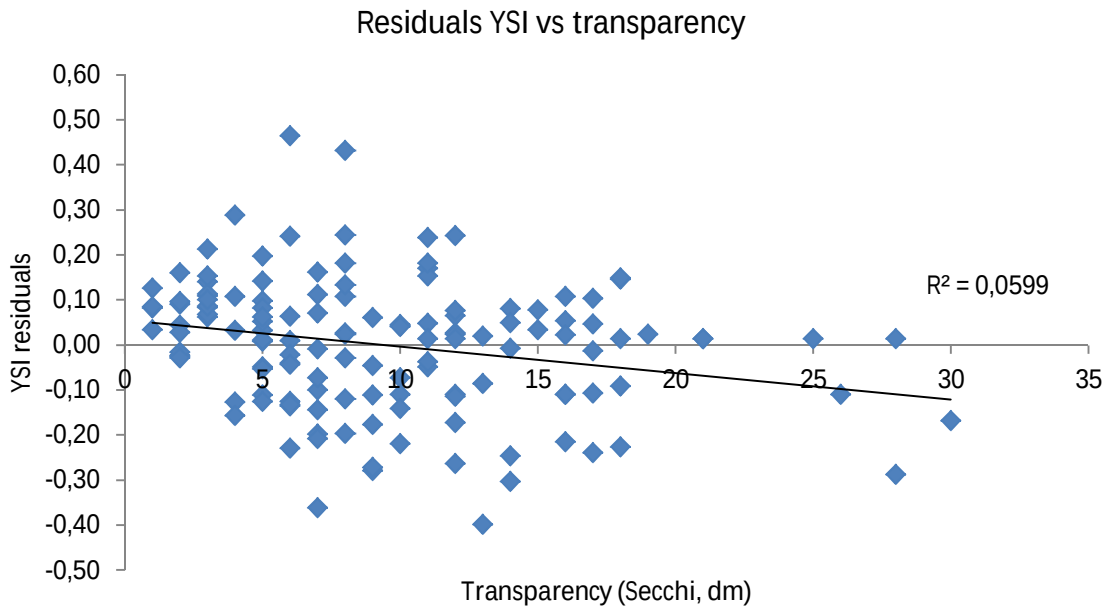

Figure 3.4 *Residuals (µg Chl/L) from the YSI fluorescence regression against NEN chlorophyll measurements (Figure 3.2) plotted against transparency measured at the time of measurements.*

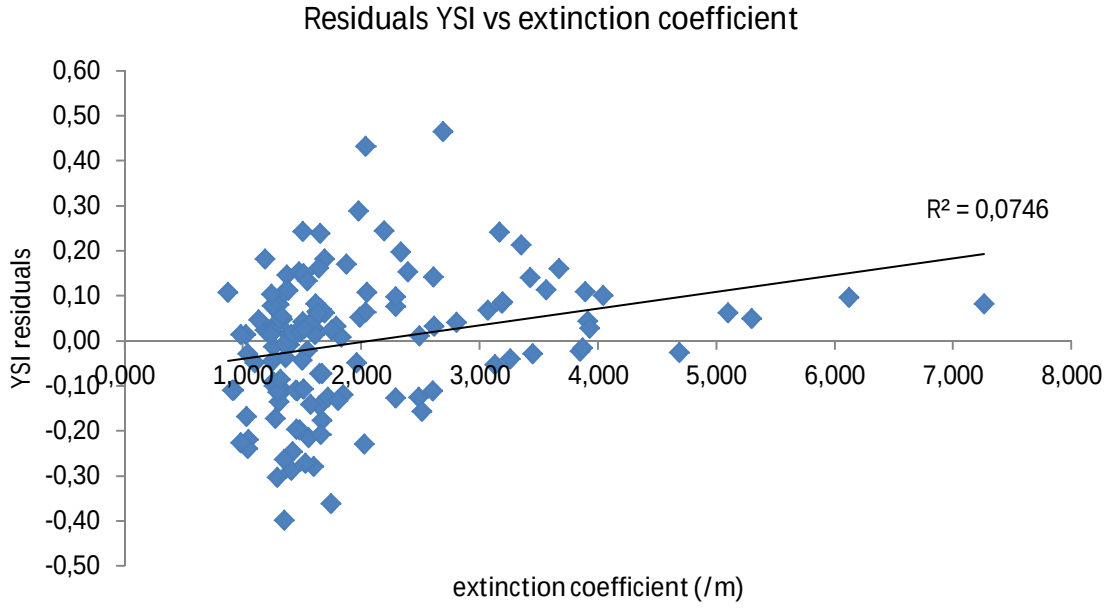

Figure 3.5 *Residuals (µg Chl/L) from the YSI fluorescence regression against NEN chlorophyll measurements (Figure 3.2) plotted against the extinction coefficient measured at the time of measurements.*

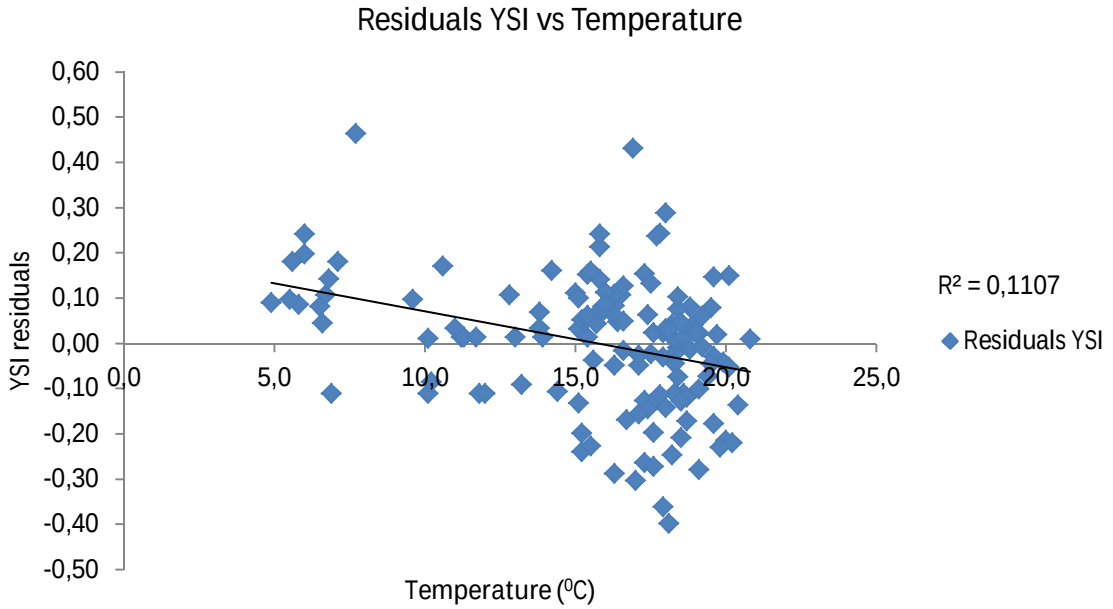

Figure 3.6 *Residuals (µg Chl/L) from the YSI fluorescence regression against NEN chlorophyll measurements (Figure 3.2) plotted against the water temperature measured at the time of measurements.*

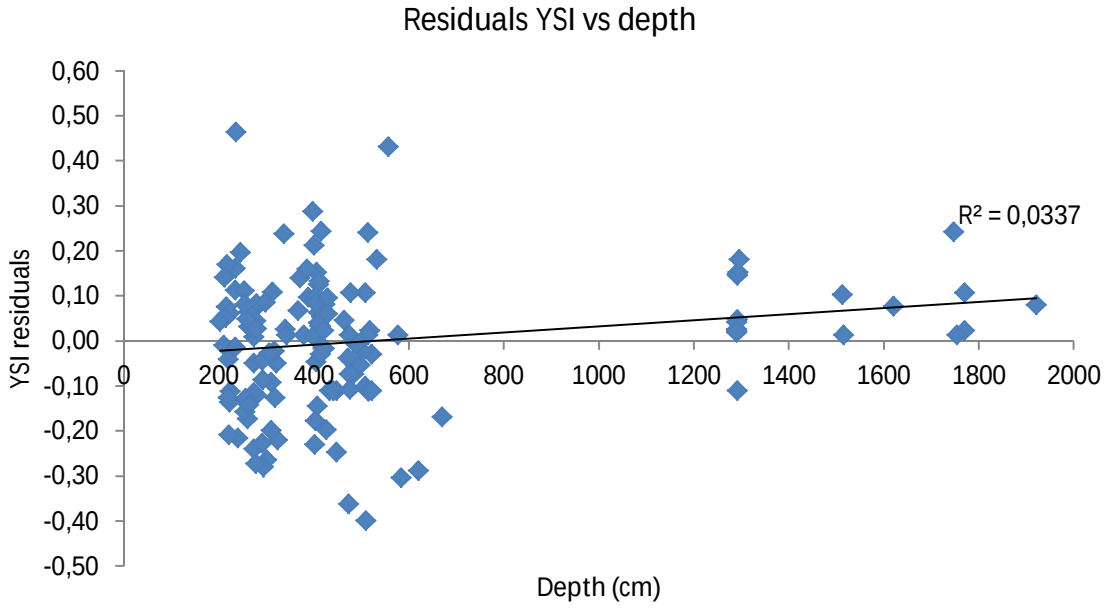

Figure 3.7 *Residuals (µg Chl/L) from the YSI fluorescence regression against NEN chlorophyll measurements (Figure 3.2) plotted against the depth of the water column measured at the time of measurements.*

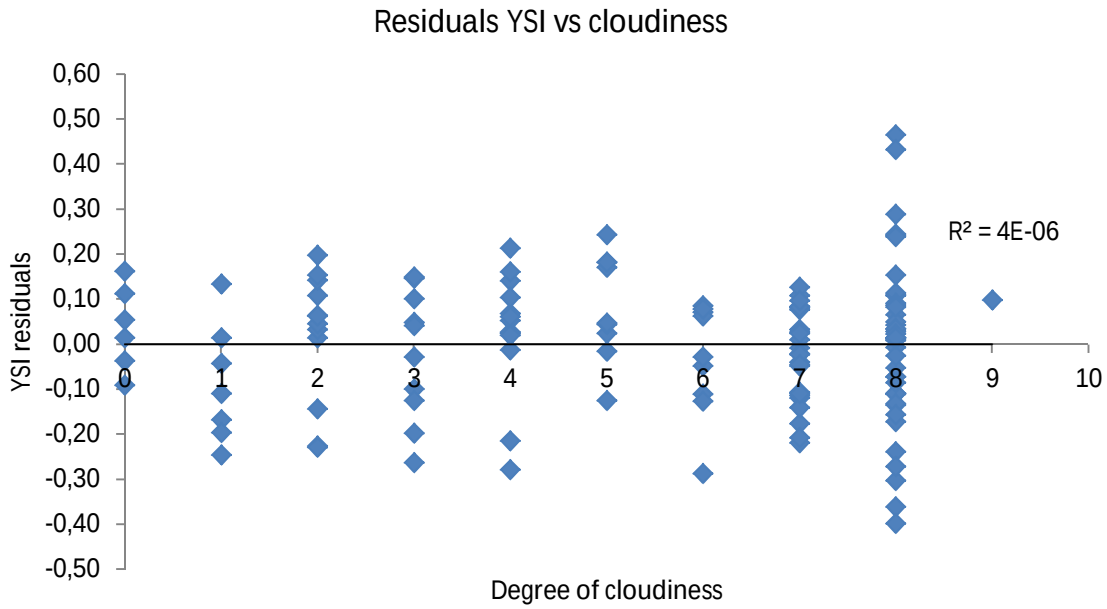

Figure 3.8 *Residuals (µg Chl/L) from the YSI fluorescence regression against NEN chlorophyll measurements (Figure 3.2) plotted against the degree of cloudiness determined at the time of measurements.*

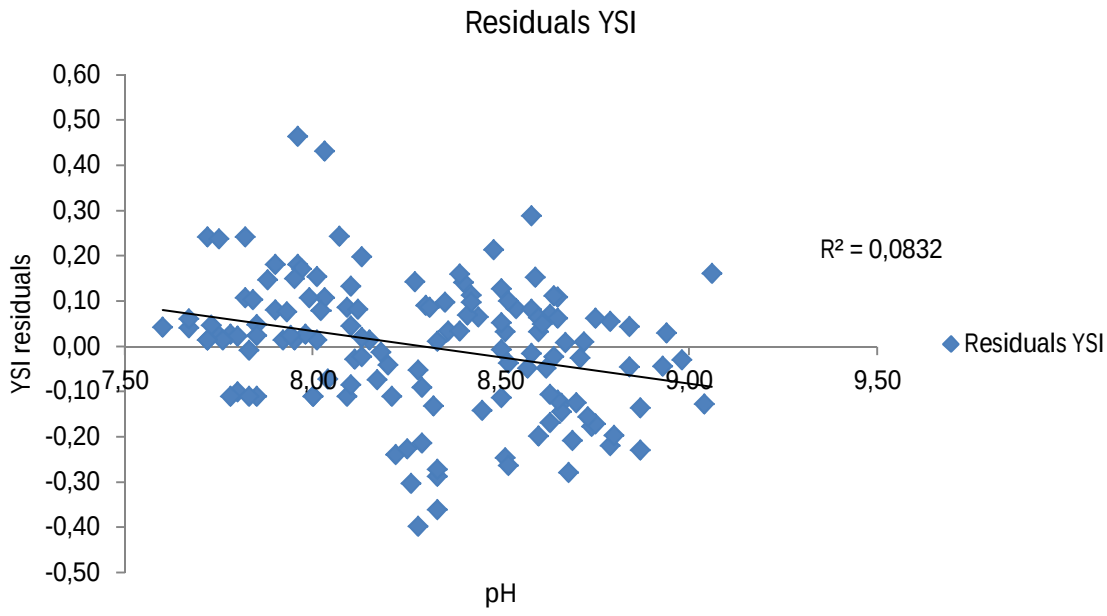

Figure 3.9 *Residuals (µg Chl/L) from the YSI fluorescence regression against NEN chlorophyll measurements (Figure 3.2) plotted against the pH determined at the time of measurements*

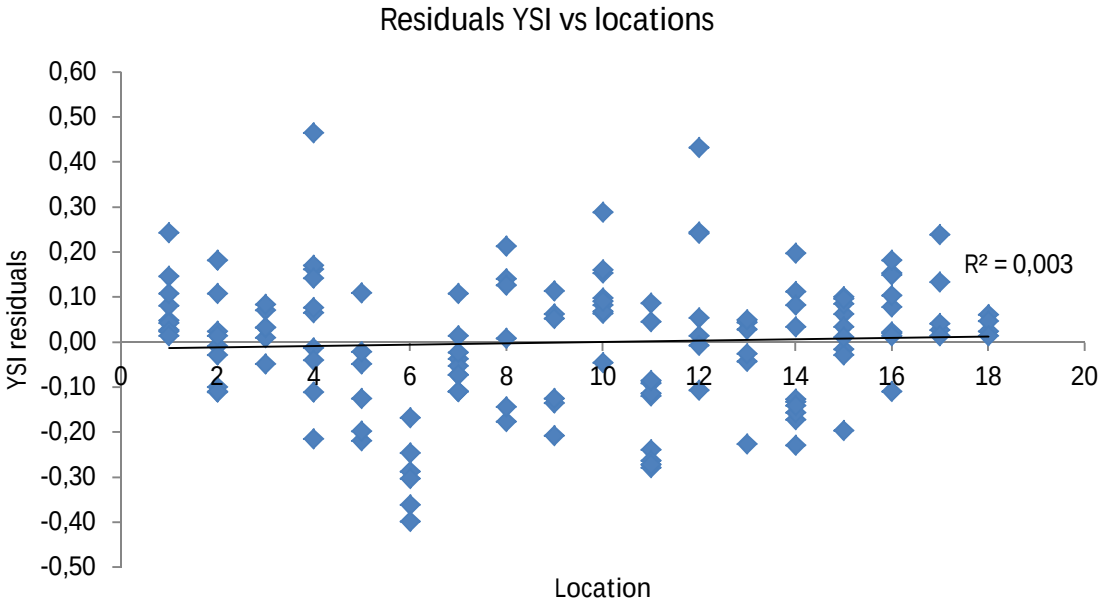

Figure 3.10 *Residuals (µg Chl/L) from the YSI fluorescence regression against NEN chlorophyll measurements (Figure 3.2) plotted against the different locations in which the measurements were performed (each location was assigned with a different number).*

None of the above mentioned factors correlated well with the YSI fluorescence, after accounting for the influence of chlorophyll (i.e. the NEN chlorophyll measurements).

The variation between NEN and YSI chlorophyll concentrations was mainly visible at low chlorophyll concentrations (Figure 3.11). Below a Box-Cox transformed NEN value of 1.50 (corresponding to an original NEN value of 5.2 µg Chl/L), the standardized difference between NEN and YSI (calculated also from Box-Cox transformed data) showed more variation than above 1.50 (0 -1.50 and 0 - 0.50, respectively). This shows that variation in measurements between YSI and NEN is mainly found at lower chlorophyll concentrations.

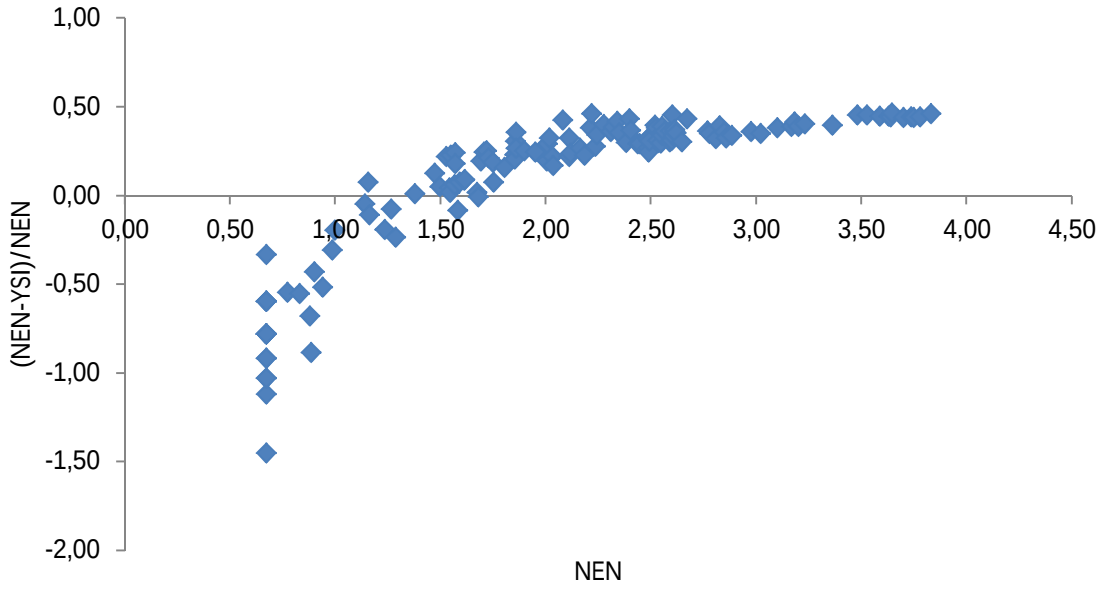

Figure 3.11 *Standardized difference between NEN and YSI chlorophyll concentrations ((NEN-YSI)/NEN) plotted against the NEN data. All data were Box-Cox transformed. A NEN Box-Cox transformed value of 1.50 corresponds to an original NEN value of 5.2 µg Chl/L.*

At the lowest measured NEN chlorophyll measurements, the YSI sensor showed variation in fluorescence results (Table 3.1). The average chlorophyll concentration measured by the YSI sensor, at NEN = 2 µg Chl/L, is 6 µg/L (original data). There is one outlier, however, (14 µg/L at location Eemmeerdijk). Therefore, in Table 3.1, the median is also presented to decrease the influence of this outlier, resulting in a value of 5 µg Chl/L for the YSI sensor at 2 µg Chl/L (as measured by NEN).

Table 3.1 YSI fluorescence results corresponding with the lowest NEN chlorophyll measurements (2 µg/L) and basic statistical results over these data. These data are original values and not Box-Cox transformed as in previous results.

Location	YSI outcome (µg/L)	Basic statistics	
Amerikahaven2	6	6	**Average**
Amerikahaven2	5	2.4	**SD**
Amerikahaven2	8	5	**Median**
Amsterdam	5	3	**Minimum**
Amsterdam	4	14	**Maximum**
Amsterdam	7		
Eemmeerdijk	14		
Houtribhoek	3		
Ketelmeer west	5		
Ketelmeer west	4		
Ketelmeer west	4		
Ketelmeer west	4		
Ketelmeer west	6		
Ramsdiep	5		
Westhaven-2	5		
Westhaven-2	4		
Westhaven-2	7		
Zijkanaal D-1	5		
Zijkanaal E	5		

If YSI and NEN data are plotted against each other (as in Figure 3.2 but then without Box-Cox transformation), then the linear regression line reads: YSI=0.6333NEN + 4.7716. At NEN = 2 µg Chl L^{-1}, this means that the YSI should equal 6.04 µg Chl L^{-1} which is more or less the same as presented in Table 3.2. At NEN = 0 µg Chl L^{-1}, YSI equals 4.77 µg Chl L^{-1}.

In Figure 3.1, eleven chlorophyll peaks are visible. For all these peaks, the YSI data are lower than the NEN results (Table 3.2). There are also other (less pronounced) chlorophyll peaks in Figure 3.1. In these cases sometimes NEN is higher than YSI but also the opposite is observed.

Table 3.2 Difference between NEN and YSI chlorophyll data for eleven chlorophyll peaks in 2011 (see Figure 3.1).

Location	Date	NEN (µg/L)	YSI (µg/L)	NEN-YSI (µg/L)
Steile Bank	24-05-2011	84.5	53	31.5
Markermeer midden	26-05-2011	99.1	74	25.1
Steile Bank	18-07-2011	67.4	33	34.4
Vrouwezand	19-07-2011	72.1	36	36.1
Steile Bank	16-08-2011	79.3	47	32.3
Vrouwezand	17-08-2011	86.5	39	47.5
Steile Bank	12-09-2011	85.4	55	30.4
Vrouwezand	13-09-2011	94	72	22.0
Vrouwezand	11-10-2011	115	66	49.0
Vrouwezand	06-12-2011	101	82	19.0
Markermeer midden	08-12-2011	106	83	23.0

When trying to relate this difference between NEN and YSI at these high chlorophyll peaks to other factors, a negative relation was found between the NEN-YSI difference and the extinction coefficient measured at the same time (Figure 3.12). The sample size was too low however, to perform regression analyses. No relation was found with other parameters measured at the same time (oxygen, temperature, transparency).

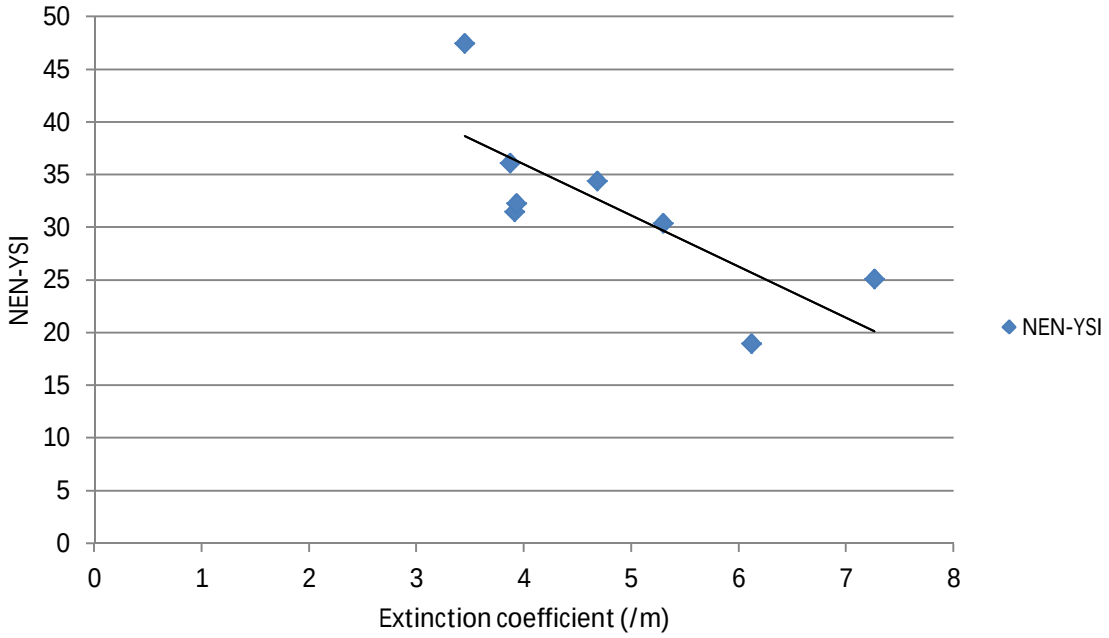

Figure 3.12 *Difference between NEN and YSI results from Table 3.2 as a function of light extinction. For Vrouwezand 13-09-2011 and 11-10-2011 and for Markermeer midden 08-12-2011 no extinction data were available. The trend line is only for visualization, data could not be transformed to obtain a normal distribution.*

From these data, it is difficult to indicate what the upper limit for chlorophyll measurements of the YSI should be. From Table 3.2 follows that 67.4 µg Chl L^{-1} (as measured by NEN), is already too high.

4 Discussion and conclusions

The research question of this study was: how much chlorophyll measurements, as determined with a fluorometer, deviate from spectrophotometric chlorophyll analyses?
Sub questions are: what are upper and lower limits of the YSI 6025 chlorophyll sensor and which factors influence possible differences between YSI and NEN measurements?

There was a good correlation between the YSI and NEN data. 72% of the variation in the YSI data could be explained by variation in the NEN data (i.e. we assume that NEN measures the true chlorophyll concentrations). Unfortunately, for the remaining 28% YSI data variation no other environmental variable was found that could explain this. There may be several explanations for this. One possibility is that there are not enough data. However, this does not seem to be the case in this study because data came from 18 locations and were measured several times during 2011. Another possibility is that there are other environmental parameters that also influence fluorescence, but were not measured. Such an important environmental factor may be the coloured dissolved organic matter (CDOM). CDOM occurs naturally in aquatic environments primarily as a result of tannins released from decaying detritus. Many inland water bodies in the Netherlands are rich in detritus and are usually coloured (yellow). CDOM contaminates the emission detection system and correcting for CDOM is very important when interpreting in situ data, otherwise chlorophyll is likely to be overestimated (Proctor & Roesler 2010). Temperature, unexpectedly with findings in literature (Roesler & Boss 2008, Proctor & Roesler 2010), did not show an effect on the YSI 6025 chlorophyll fluorescence outcomes. One reason for the lack of a temperature effect on chlorophyll fluorescence readings on the YSI 6025 sensor may be that this sensor was already corrected for temperature effects (as is explained in the manual of YSI). If the sensor has been temperature corrected is unknown to Deltares.

Another possible source of influence on in vivo chlorophyll fluorescence is the (variation in) algal composition. Different phytoplankton groups arise at different times during the season. For instance, diatoms usually arise in early spring while cyanobacteria become dominant during summer. An important aspect here is that, although all phytoplankton contains Chl *a*, they very much differ from each other in their accessory pigment composition (Sathyendranath et al. 1987). These different pigments contribute differently to absorbed excitation energy which leads to different chlorophyll fluorescence excitation (Poryvkina et al. 2000). It is therefore important that when in vivo chlorophyll fluorescence is determined, also information on the phytoplankton composition is obtained. Several authors state that fluorometers, like the YSI 6025 sensor, should be frequently calibrated with the natural phytoplankton assemblage of the location of interest (Lawrenz & Richardson 2010; Richardson et al. 2010). Furthermore, in cells the pigments are packaged after formation. This package results in pigments ‘shading’ each other from detection (the fluorescence efficiency of each chlorophyll molecule decreases then, Morel & Bricaud 1981) and in photochemical and non-photochemical quenching of fluorescence (Proctor & Roesler 2011).

The output of the YSI 6025 sensor is read either as relative fluorescence units (RFU) or chlorophyll concentration (in µg/L). The RFU are the raw measuring data while the chlorophyll readings are derived from the RFU data. It is unclear, however, how chlorophyll concentrations are derived from the RFU data. Therefore, the fact that the chlorophyll data are derived data may also be a factor that leads to differences between YSI and NEN.

As a suggestion, the RFU output of the YSI 6025 sensor could be calibrated against the NEN output to check if the chlorophyll output of the YSI sensor is correct.

At the lowest measured NEN values, the median YSI measurements were 5 µg Chl L^{-1}. This is somewhat higher than the lower limit of the spectrophotometric analyses (NEN; 2 µg Chl L^{-1}). A possible explanation may be that after cell lysis, the chlorophyll pigments (because they are not water soluble) are still capable of light absorption and excitation (Lurling & Verschoor 2003). This does not happen during in vitro analyses so it is possible that at 2 µg Chl L^{-1}, as measured in vitro (NEN), the in vivo analyses (YSI) gave higher chlorophyll readings because they also measured chlorophyll pigments originating from dead cells. The YSI value of 5 µg Chl/L however, does not mean that this is also its lower limit. It is the lowest value in the comparison with the NEN data. It looks as if the YSI is as sensitive as the NEN method, although the YSI output is higher. It may also be that the lowest NEN data are not correct. Deltares advices to compare NEN and YSI using a serial dilution. This should be done preferably with water from the location of interest if information of the algal composition is known. Otherwise laboratory cultured algal strains should be used.

The upper limit of the YSI 6025 sensor was difficult to establish. At times of high chlorophyll concentrations (as measured spectrophotometrically), the YSI measurements were always lower (Figure 3.1). A possible explanation for this may be a shading effect between cells when concentrations are high. This influences in vivo chlorophyll fluorescence (Proctor & Roesler 2010) but not in vitro because the latter technique involves extraction steps of samples, which eliminates cell density problems. Another possibility is that under conditions of low light levels (which may happen when the phytoplankton biomass is high) cells produce more chlorophyll (Cullen & Lewis 1988). However, the fluorescence yield per Chl *decreases* because chloroplasts are less efficient. In this case, there is more chlorophyll, which is detected spectrophotometrically but less fluorescence, as detected by the YSI. It is therefore important that also information on the phytoplankton assemblage and biomass is obtained during YSI chlorophyll fluorescence measurements.

Overall, the YSI 6025 sensor for in vivo chlorophyll measurements gave a good correlation with in vitro chlorophyll measurements (NEN). This seems promising for a future application in field monitoring by Rijkswaterstaat if the remaining variation in fluorescence in the YSI can be identified and quantified. Which other sources contributed to this fluorescence variation remains unknown but it is strongly suggested to include CDOM and algal composition (and concentration) in future field surveys with the YSI 6025. This does not mean that the other parameters investigated in this study, should not be measured anymore. As said before, other studies have indicated that temperature did indeed have an effect on chlorophyll fluorescence and also YSI self states this (not an effect on the sensor itself but on the fluorescence of the phytoplankton suspensions) (YSI-6-series Manual September 2009). It therefore remains important to continue the monitoring with the parameters that were used here added with CDOM and algal composition and concentration.

Finally, YSI warns researchers that in vivo fluorescence, as done with the YSI 6025 sensor, will never replace the standard procedures as NEN. Rather, a fluorometer should be used to complement the more accurate but more difficult to obtain data from the standard procedures.

Conclusions

- Chlorophyll fluorescence as measured by the YSI 6025 chlorophyll sensor correlated well with spectrophotometric measurements.

- The remaining variation in fluorescence could not be explained by variation in other measured environmental variables (e.g. temperature, suspended solids concentration, transparency) (and may be the result of the sum of little variation of the individual parameters.)
- The lower limit of the YSI 6025 chlorophyll sensor at which it can be compared to the NEN-method is 5 µg Chl L^{-1}. Deltares advices to compare NEN and YSI using a serial dilution. This should be done preferably with water from the location of interest if information of the algal composition is known. Otherwise laboratory cultured algal strains should be used.
- The upper limit of the YSI 6025 chlorophyll sensor could not be established with the current data set. At high chlorophyll peaks, the YSI sensor always resulted in lower chlorophyll concentration estimates than the NEN analyses. This is probably the result of shading effects and lower fluorescence yield per Chl *a* under low light conditions.
- The YSI 6025 sensor seems a promising tool which can be used complementary to standardized spectrophotometric analyses. To find out what the unexplained variation is between the YSI and NEN, Deltares advices to include CDOM and algal composition (and concentration) in the field monitoring program.
- To obtain correct chlorophyll readings, the YSI 6025 chlorophyll sensor should be calibrated with the phytoplankton assemblage of the location of interest.

5 References

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