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The Use Of Pulse Amplitude Modulation (PAM) Fluorescence Technique to Assess the Periphyton Community

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Abstract

Microphytobenthos provide an energy source for other organism in the running water ecosystems. As they are attached on the substrates in the stream and river, they are seen as a good indicator for environmental monitoring in aquatic ecosystems. To assess changes of benthic algal community, the abundance, species number and the pigment content are metrics useful to observe environmental changes. In order to analyze for example the pigment content, fluorescence based measurements are suitable method. Pulse Amplitude Modulated (PAM) fluorescence techniques is the fast, non-destructive tools that have been used to measure the chlorophyll fluorescence and photosynthetic rates in algae. Therefore, this thesis was investigated the use of PAM technique to observe the periphyton community in term of biomass contents and class composition. Also analyzing the case study of hydro-peaking effects experiment using PAM measurement, in collaboration with the use of other methods (HPLC, Fluorometry). The findings confirmed that PAM could be an authentic tool to assess the patterns of benthic algae structure changing over time, enabling rapid evaluation. Moreover, using PAM has to take into account about the thickness and type of samples; also the combined methods should be required for more productive measurement.

Introduction

In many ecosystem studies, the biomass of phytoplankton or phytobenthos is used to get information about the trophic state or the effect of different environmental factors on the system. Microphytobenthos for example are important primary producers in low order streams, and therefore form the base for the food web (Allan J.D et al. 2007; Stevenson R.J et al, 1996). The majority of species found within the microphytobenthos belongs to the group of Cyanophyceae (Sachs, 1874), Chlorophyceae (Warming, 1884) and Bacillariophyceae (Dangeard, 1933). The distribution and composition of microalgae in natural habitats has been tightly linked to environmental factors (Round, 1981; Van, 2004). Therefore, they have become a useful indicator of stream water quality as they provide a continuous record of environmental quality. Short life cycles allow them to response fast to the changes of environmental factors, such as nutrient enrichment, temperature changes, light intensities and toxicity. They are also easy to handle and curate as a sample, besides they can be stored in only small space, facilitate long-term storage

(Stevenson R.J et al, 1996) This is why they are ideal organisms to use in water quality monitoring.

In these studies the chlorophyll a content as a major light harvesting pigment for photosynthesis is often used as a surrogate parameter for algal biomass. It is not only important to have the information on the biomass but also how its composition is, in order to study the community structure. Therefore, the accessory pigments (e.g. chlorophyll b and c, carotenoids) can also be used to get a more detailed picture about the biomass and the algal composition. Algal classes are characterised by specific marker pigments of antenna complexes of photosystem II (PSII), which can be used to assess the distribution of an algal class within succession of algal community, PSII is also important for the fluorescence detection (Ruser et al, 1998). Thus, it is extremely difficult to adequately sample populations of microalgae and there is requirement of differentiating algal populations, also the development of alternative methods to detect changes of microalgae community structure has been a challenge. According to that, the unique optical properties of chlorophyll a and the accessory pigments have been used to develop spectrophotometric (Jeffrey & Humphrey, 1975) and fluorometric (Holm-Hansen et al, 1965) measurement techniques. The disadvantages of most of these methods are based on the fact that sample processing is time consuming and the samples have to be handled quite carefully, as the chlorophylls are easily destroyed by light and/or heat. Nowadays, the High Performance Liquid Chromatography (HPLC) analysis, which used in this study, is considered the best method. In addition to chlorophyll a, it allows the determination of the most important photosynthetic pigments and their derivate (Mantoura & Llewellyn, 1983). However, the disadvantages mentioned before are also applied (sample processing, no online-measurements possible).

Therefore, methods for in-situ measurements of chlorophyll a and the accessory pigments were developed. This thesis focuses on the Pulse Amplitude Modulation (PAM) technique, which is a variable fluorescence measurement. Fluorescence is the re-emission of the light photon, with a lower energy than the photon absorbed. Most of the fluorescence that we measure comes from the light-harvesting complexes of PSII (Consalvey M. et al, 2005). There are a variety of PAM fluorometers now available for use in the laboratory as well as in situ and even underwater, whereas in this study particularly used Phyto-PAM (Heinz Walz GmbH, 2003), which is the ultrasensitive four-wavelength (470, 520, 645 and 665 nm) chlorophyll fluorometer. The fiber version was used in this case as it provides a focusing fiber optic for microphytobenthos measurements. This feature is very useful for distinguishing algae with different types of light harvesting pigment antenna.

Consequently, this technique has been used to measure algal photosynthesis and chlorophyll a concentration via employed light emitting diodes (LED) to excite chlorophyll fluorescence by light pulses at different wavelengths, although mainly on cultures (Barranguet et al. 2000; Kroon et al. 1993; Hofstraat et al. 1994; Kroon, 1994; Geel et al. 1997; Flameling & Kromkamp, 1998). The Phyto-PAM not only allows determining the content of active chlorophyll, but also differentiating between different pigment groups of algae (green algae, diatoms and cyanobacteria). In addition, PAM could assess the light-adaptation state of the various types of algae; also it could instantly calculate the Electron Transport Rate (ETR), which can be used to examine microphytobenthic photosynthetic activity. Previously Van (2004) had applied the use of Phyto-PAM to study the biomass changing over time, comparing to the use of extraction method and the results showed that there was the similar growth curve between two methods. Moreover, they have been successfully used PAM to study the composition of algal community, also measured photosynthetic capacity. And according to (Consalvey M. et al, 2005; Serôdio et al. 1997, 2001; Barranguet et al. 2000; Honeywill 2001; Honeywill et al. 2002) they stated that using the minimum fluorescence (F_o) as an indicator of the biomass reported a linear relationship between F_o and chlorophyll a (as a proxy for microphytobenthic biomass) in the laboratory and in situ. PAM fluorometry is a very useful tool for community level multi-parameter assessment of biofilms. The method reveals a good discrimination of major microphytobenthic groups and was able to detect changes in community structure. It also allows rapid analysis of the photosynthetic quantum yield as a functional parameter of microalgae (Schmitt-Jansen et al, 2007). This equipment enables not only a determination of the whole biofilm but also the photochemical behaviour of individual cells within intact biofilm (Oxborough et al, 2000; Perkins et al, 2002). In the studies of algal photophysiology and for the measurement of algal production rates, fluorescence is used as rapid, non-destructive proxy of photochemical efficiency and photosynthetic rate. It is also increasingly being used as an ecological tool, which based on the fluorescence properties of PSII.

PAM demonstrates clear advantages of its rapid, non-invasive (enabling repeated measurements) technique and also able to obtain in situ. On the other hand, there are some difficulties working with PAM fluorometer on benthic biofilms: the signal measured by a PAM is depending on the sediment optics, the 'measuring depth' of the modulating light will depend on the (absorption by the) algal biomass, its vertical distribution and sediment characteristics such as grain size, water content, and organic matter content (Kromkamp et al, 2006). When the sediment depth is high or the algal growth is older (thick surface) the light pulses could not detect accurately and might

miss the important information during the measurement. Therefore, using PAM would be more suitable for the initial phase of algal growth.

Accordingly, this thesis aimed to find an adequate calibration for the Phyto-PAM to measure the chlorophyll a concentration and also to differentiate three algal groups. Furthermore, the use of this technique for a hydropeaking study is shown as an example. Regarding to that, we hypothesized that using PAM measurement technique gives a reliable result in a rapid and non-destructive way, and there is a correlation of chlorophyll a concentration between the two methods (Phyto-PAM VS HPLC; PAM VS Fluorometer). In addition, under the hydropeaking events, the community structure changes.

Material and methods

Algae group detection by Phyto PAM

The Phyto-PAM (Heinz Walz GmbH) was used for fluorescence measurement, which equips with a special fiberoptics-Emitter-Detector-Unit, PHYTO-EDF. The fluorescence pulses are detected by a photomultiplier and amplified under microprocessor-control, resulting in four separate continuous signals. The fluorometer is operated in conjunction with a computer and special Windows software for data deconvolution and analysis. The measurement involved a chlorophyll determination in µg/cm^2 on the basis of chlorophyll fluorescence yield in the quasi-dark state (Fo) and determination of the quantum yield of photosystem II with the help of a brief pulse of saturating light. When a saturation pulse is triggered, the momentary fluorescence yield (Ft) is sampled and the increase of fluorescence (dF=Fm-Ft) is determined. The quantum yield (Yield) corresponds to dF/Fm. The displayed data for cyanobacteria (Blue), green algae (Green) and diatoms (Brown) were calculated from the original four-channel fluorescence data by an on-line deconvolution routine, based on previously stored 'reference excitation spectra' or mostly called 'References'. This "spectra" that consist of four points at 470, 520, 645 and 665 nm, can be readily measured under "Reference" for each algae group (Ulrich, 1998). These fingerprints from each group will be stored in separate file. Detail for Phyto-PAM methods (Heinz Walz GmbH, 2003).

Preparation for the calibration and reference spectra

For the calibration pure samples of three different algal groups were used (Cyanophyceae, Chlorophyceae and Bacillariophyceae). As a representative for the Cyanophyceae, we used a

culture of *Synechococcus sp*., and for the Chlorophyceae was the culture of *Scenedesmus sp*.. The algal cultures are kept at 22 °C, the photoperiod is 14:10 (light:dark) and under the wc modified medium. Bacillariophyceae samples were taken from the outflow of Lake Lunz. After collecting samples, microscopic classification was used to check for the clarification on species.

Phyto-PAM measurement

References were measured from each algal group: green, blue-green and brown algae, respectively. In order to measure references, we pipetted 200 µl of each sample into the small ring area of 1,131cm^2 on the slide and measured with Phyto-PAM. The data then stored under references of each algal group. Meanwhile, the calibration also had been made, particularly, the calibration had been made for two types of samples, water and stone. For the water samples, with the same sample that measured for the references, we calibrated against three algal groups after known the chlorophyll a concentration measured with fluorimeter. Besides, stone calibration also made by filtrated 5ml of each algal group sample on the filter and measured with Phyto-PAM on the filter surface and calibrated three algal groups later, when known the chlorophyll a concentration from fluorimeter measurement.

Acetone based chlorophyll-a determination

To know the certain concentration of the calibration samples, the chlorophyll a content was measured by acetone extraction and measurement with the fluorimeter. Samples were filtrated through GF/C filters (Whatman) with sufficient amount until some colours appear on the filters, afterward the filter was cut into different glass vial, filled 5-6ml of acetone with dispenser, put the samples in the white box to avoid the light and sonicated at 30% amplitude, for 30 seconds (1sec./1sec.). After that, the samples were kept in the fridge at 4° for 6-16h. After the extraction in acetone, samples were centrifuged at 2500rpm for 10 minutes and immediately measured for chlorophyll a concentration with fluorimeter. The Chlorophyll a was then calculated with these following formulas:

$$Chla0-400\left[\mu g\cdot l^{-1}\right]=\frac{((1{,}3451\cdot em)-1{,}7101)*Vol.extract[ml]}{Vol.filtrated[ml]}$$

$$Chla0-1000\left[\mu g\cdot l^{-1}\right]=\frac{((1{,}3954\cdot em)-3{,}9944)*Vol.extract[ml]}{Vol.filtrated[ml]}$$

HPLC measurements

The preparation of samples for HPLC was done the same step as fluorimeter. Therefore, after extraction, 1ml of each sample was filled in HPLC vials and they were analysed by HPLC according to (Woitke et al. 1994) described methods for classification of algal group and chlorophyll a concentration. After knowing all the pigments in the samples from HPLC then the results analysed by CHEMTAX195 (Mackey M. D. et al. 1996) according to pigment ratio matrix (Table.1). The biomarker pigment ratio to chlorophyll a were used to support the initial matrix needed for CHEMTAX analysis, where the software was run to estimate the biomass of diatoms, green algae and blue-green algae which were expressed as chlorophyll a.

Algal group	Biomarker pigment ratios to Chl a									
	alx	**chb**	**dia**	**din**	**echi**	**fucox**	**lut**	**per**	**zeax**	**chla**
Chlorophytes	0	0,406	0	0	0	0	0,165	0	0,008	1
Cyanobacteria	0	0	0	0	0,056	0	0	0	0	1
Diatoms	0	0	0,278	0	0	0,738	0	0	0	1

Table.1 CHEMTAX pigment ratio matrix used in Chemtax (Browne, Jamie L., 2010)

Validation of the calibration and references

For the validation, a dilution row was performed from each algal group (Table.2; Table.3; Table.4; Table.5), as well as the mixed cultures (Table.6; Table.7). The samples included 10 of green algae, 10 of blue-green samples, 10 of diatoms and 10 of mixed cultures. Notably, the samples we used for green and blue-green algae were cultures from WLC and diatoms were taken from the outflow of Lake Lunz. Furthermore, we also included 10 samples from mixed stone, which collected from natural sites. These stones were scraped their attached algae on its surface and mixed with MiliQ water. These samples were categorized in the water samples and each of them was pipetted into small ring slide and measured with Phyto-PAM under water calibration. In addition, 10 stone samples collected from the outflow of Lake Lunz also included in the measurement but with the different step. After taken all stones to the lab, we measured with Phyto-PAM directly on stone surfaces under stone calibration. After measure with Phyto-PAM, the algae was removed from the surface of the stones by spatula and dissolved in MiliQ water. Lastly, samples were also measured with fluorimeter for chlorophyll a total and HPLC for composition group (the step applied as described above). Markedly, all measurement with Phyto-PAM was done under the same references we made.

Sample#	1	2	3	4	5	6	7	8	9	10
Blue-green (ml)	10	9	8	7	6	5	4	3	2	1
Water (ml)	0	1	2	3	4	5	6	7	8	9

Table.2 Dilution row of blue-green algal samples

Sample#	1	2	3	4	5	6	7	8	9	10
Green (ml)	10	7	6	4	3	2	1	11	10	5
Water (ml)	0	3	4	6	7	8	9	2	2	7

Table.3 Dilution row of green algal samples

Sample#	1	2	3	4	5
Diatoms (ml)	10	5	6	8	9
Water (ml)	0	5	4	2	1

Table.4 Dilution row of diatom I samples

Sample#	6	7	8	9	10
Diatoms (ml)	5	6	7	8	9
Water (ml)	5	4	3	2	1

Table.5 Dilution row of diatom II samples

Sample#	1	2	3	4	5	6	7
Green (ml)	1	5	3	2	2	2	3
Blue-green (ml)	5	2	1	4	2	5	3
Diatoms (ml)	4	3	6	4	6	3	4

Table.6 The amount of the mixture in mixed cultures

Sample #	8	9	10
Mixed algae (ml)	10	5	7
Water (ml)	0	5	3

Table.7 Dilution row of samples mixed from diatom-green algae samples

Existing data set from the experiments in 2014

The existing data from previous experiment in summer 2014, at HYTEC (Hydromorphological and Temperature Experimental Channel) were computed with new calibration and references. The data contained the results from measurement with Phyto-PAM regards the effects of hydropeaking on periphyton community. It consisted the data from with and without effects of hydropeaking, included total chlorophyll a concentration and chlorophyll a of each algal group – the brown, blue-green and green algae. We analysed the differentiation of chlorophyll a concentration between two conditions of hydrological changes in two habitat types – pool and

riffle, as well as the composition changes. Besides, the samples from the same experiment, which kept in the freezer, were also measured for chlorophyll a concentration with fluorimeter and HPLC. In contrast, we have to take into account that the results were not comparable since the samples collected differently. As PAM measurement was done the day before with two stones in each sub-site, later the samples were put together and the next day, samples were collected from one stone and kept for further analyses.

Results

In order to evaluate the use of Phyto-PAM measurements for the determination of microphytobenthos biomass and group classification, several calibration and references measurements were performed. Two approaches were used; firstly, cultures for each algal group, defined mixed cultures and natural mixed stone samples were used to see if the chlorophyll a calibration worked well. Secondly, stone samples were measured by the Phyto-PAM directly on the surface of the stones. Besides, the samples were also analyzed with HPLC and fluorimeter.

Total Chlorophyll a calibration

So simulate natural conditions, mixed samples from the cultures were produced. The detailed description of the used ratios from the three different groups is listed in (Table.6) and for mixed diatoms and green algae is list in (Table.7).

Looking at the comparison of Phyto-PAM measurements with the acetone based fluorimetric analyses, the total chlorophyll a content showed a linear correlation with an r^2 of 0.687 (Fig.2). However, the Phyto-PAM overestimated the content up to 56%. The same procedure was carried out with natural samples, scraped from 10 stones found in the outflow section of Lake Lunz. These scraped stone samples showed also a linear correlation with an r^2 of 0.516. Again the Phyto-PAM overestimated up to 60 % (Fig.3)

The last step for testing the quality of the conducted chlorophyll a calibration was to measure directly on the surface of natural grown periphyton on stones. Here, the results showed a totally different picture, we found no linear correlation ($r^2 = 0.142$), and the Phyto-PAM underestimated the total chlorophyll a values up to 86 % (Fig.4).

Determination of three major benthic algal groups

Concerning the chlorophyll a determination of three different algal groups, the results showed a very good correlation between the Phyto-PAM measurements and the acetone extraction based fluorometric analyses. The blue-green algal group showed an r^2 of 0.97 (Fig.1), however, the Phyto-PAM (chlorophyll a conc. ranged from: 0.07-0.46 µg/cm^2) showed up to 46% higher values than the classical fluorometric method (Chl a values from: 0.03-0.26 µg/cm^2). The brown algal group gave a linear correlation with an r^2 of 0.823 (Fig.1) and also in this group the Phyto-PAM overestimated in the mean with 32% (Chl a values: PAM: 0.05-0.14 µg/cm^2; fluorimeter: 0.03-0.08 µg/cm^2). The green algal group also showed the good correlation with an r^2 of 0,758 (Fig.1) and again Phyto-PAM (Chl a: 0.21-1.56 µg/cm^2) overestimated 25% compared to the results from fluorimeter method (Chl a: 0.16-1.52 µg/cm^2).

To evaluate the ability of the Phyto-PAM to detect the different algal groups in one sample, we mixed the different cultures in a certain ratio (Table.6; Table.7). In addition, we also measured these samples with the HPLC method, to see which method delivered more reliable results. In general, results from PAM showed better detection compared to the percentage of proportion known from cultures (Table.8). PAM illustrated 50% of culture mixed samples with the right detection, while only 20% of results from HPLC matched with the real known composition (Table.8). In addition, sample 1 to sample 7 were the mixtures from three algal groups (blue-green, green, brown); both PAM and HPLC gave only two correctly detections, while in these two, both methods shared only one same sample which was the sample 7 (Table.8) where they detected blue-green, green and brown algal groups. Moreover, when we focused more into majority of community, in this sample, regarding to the cultures mixed, they were dominant by green algae (82%), then blue-green algae (14%) and last one was diatom (4%) (Table.8). Thus, PAM showed more suitable pattern to the known community structure (%green: %blue: %brown) (PAM – 77%: 15%: 8%), while the community dominance was slightly different according to HPLC (HPLC – 75%: 11%: 14%) where diatoms made up more percentage than blue-green algae (Table.8). Furthermore, sample 8 to sample 10; they were the diatom mixed green algae samples with different portions mixed with water (Table.7). Here we did not know the exact percentage of each group in the mixtures but there were surely only diatom and green algae, hence, PAM showed all samples accurate detection while none of HPLC's results did (Table.8).

Example from the field - Effect of hydropeaking on the succession of benthic algae

The measurement of chlorophyll a concentration under the effect of hydro peaking experiment was done by phyto-PAM; the existing data were then updated with new calibration and investigated if it could be useful tool, in comparison to the patterns of changing from the similar samples measured by fluorimeter and HPLC. In consideration, both samples were collected in the different time and different amount. When PAM measurement was done, we measured randomly on some stones in the community and later the samples for fluorimeter measurement were collected from one stone, according to this the results would not be exactly the same. In this case, we only compared and studied the pattern of growth within the community of periphyton under hydro-peaking stress.

Application to assess the biomass changing pattern

In order to test whether the PAM measurement could be used to observe the changes in benthic algae community over time, we analyzed the data from the case study of the previous experiment. The results from the fluorimeter and the Phyto-PAM showed slightly different pattern of growth when there was no hydropeaking (Fig. 5; Fig.6). In the hydropeaking treatment, the changing patterns were similar (Fig.5; Fig.6). The biomass loss could be seen after 53 days of the experiment especially in the riffle habitat, where the biomass was lost from 1.02µg/cm^2 to 0.70µg/cm^2 (PAM) and for the changes measured by fluorimeter, the biomass decreased from 1.80 to 0.91 µg/cm^2. In pool habitat, only the fluorimeter showed the loss of biomass (0.75 to 0.52 µg/cm^2). Additionally, both methods showed from day 19 until day 46 that the biomass increased in the hydropeaking treatment and after 46 days the biomass started to decline. The only different that could be noticed was in pool habitat, where PAM measurement specified that starting from day 19 in the hydropeaking treatment, there was biomass loss from 0.16 to 0.03 µg/cm^2 and after 19 day the growth was increasing until the last measurement (Fig.6). In addition, the data from PAM measurement was more variable (Fig.6).

Application to detect population composition changing

The population differentiation of the microphytobenthos over time measured by PAM showed that there were more changes of majority population in the hydropeaking treatment (Fig.7; Fig.8). Starting at day 19 of the experiment, at pool habitat the main composition dominated by 46% of blue-green algae, second majority was diatoms (29%) and the rest were green algae. In contrast,

with the hydropeaking event the main population was green algae that made up for 56% of the population, 28% was diatom, and the blue-green algae was lost its biomass to only 17% (Table.9). Moreover, at riffle the majority changed from diatoms (57%) to blue-green algae (56%) (Table.9). The next date of the measurement was on day 40 of the experiment; the proportion of community did not show huge difference. At pool, the main composition was blue-green algae (%blue: %green: %brown) (67:3:30) and after the event, it still remained (64:5:30). At riffle, blue-green algal was also still the dominance of the community (74:0:26), and after the peaking-event, blue-green algal was still the majority but with the existence of green algae (68:6:26) (Table.9). Furthermore, the last date of the measurement was on day 53 where in the hydropeaking treatment, the community endured similar structure with the main proportion of blue-green algal and the second main composition was diatoms at both pool and riffle (Table.9) with the loss of green algal.

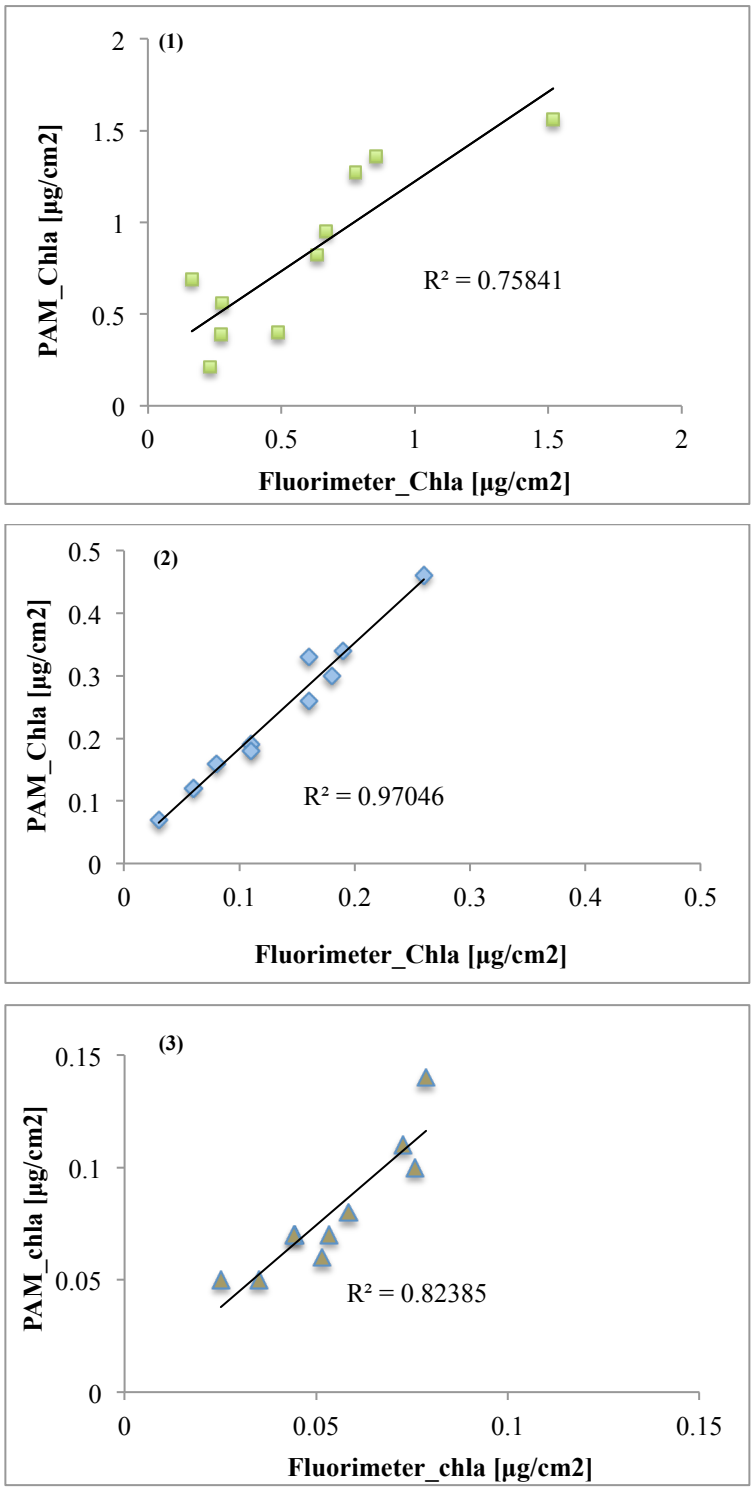

Fig.1 Correlation between PAM and fluorimeter measurement for Chl a of pure algal samples, 10 samples for each algal class - Green (1), Blue-green (2), Brown (3)

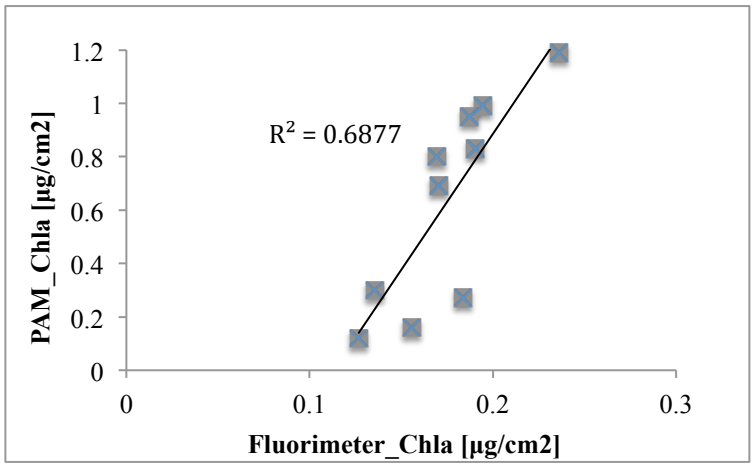

Fig.2 Correlation between PAM and fluorimeter measurement for total Chl a of 10 mixed cultures

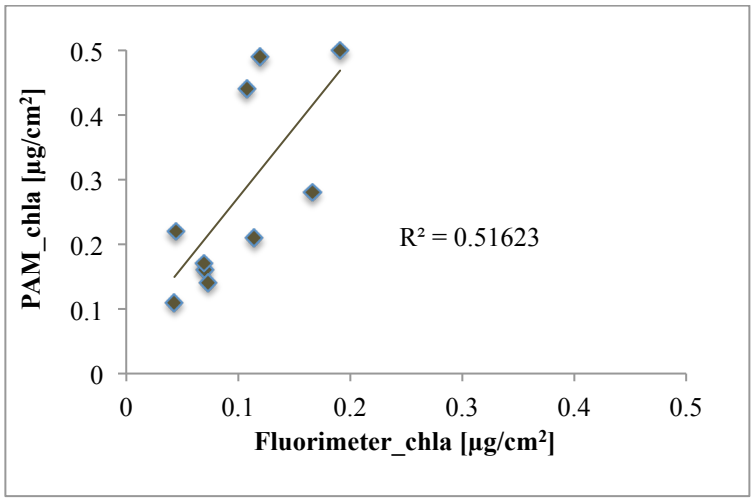

Fig.3 Correlation between PAM and fluorimeter measurement for total Chl a of 10 mixed natural stones

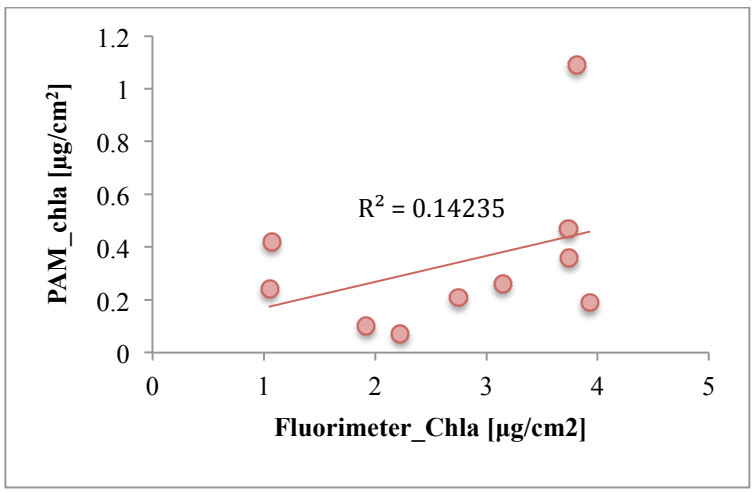

Fig.4 Correlation between PAM and fluorimeter measurement for total Chl a of 10 stone samples (surface measurement)

% of algal class in Mixed Cultures

Sample	Known % from cultures mixed				PAM			HPLC		
	% Blue	% Green	% Brown	% Green+Brown	% Blue	% Green	% Brown	% Blue	% Green	% Brown
1.M	42	48	10		57	43	0	0	84	16
2.M	6	91	3		8	92	0	0	98	2
3.M	5	86	9		0	87	13	0	82	18
4.M	24	69	7		44	56	0	0	85	15
5.M	13	78	9		20	62	17	0	77	23
6.M	29	67	4		48	52	0	3	85	12
7.M	14	82	4		15	77	8	11	75	14
8.M				100	0	63	37	30	57	13
9.M				100	0	92	8	43	47	10
10.M				100	0	75	25	35	54	11

Table.8 The percentage of the algal group in the mixed culture samples – known %, % from PAM, % from HPLC

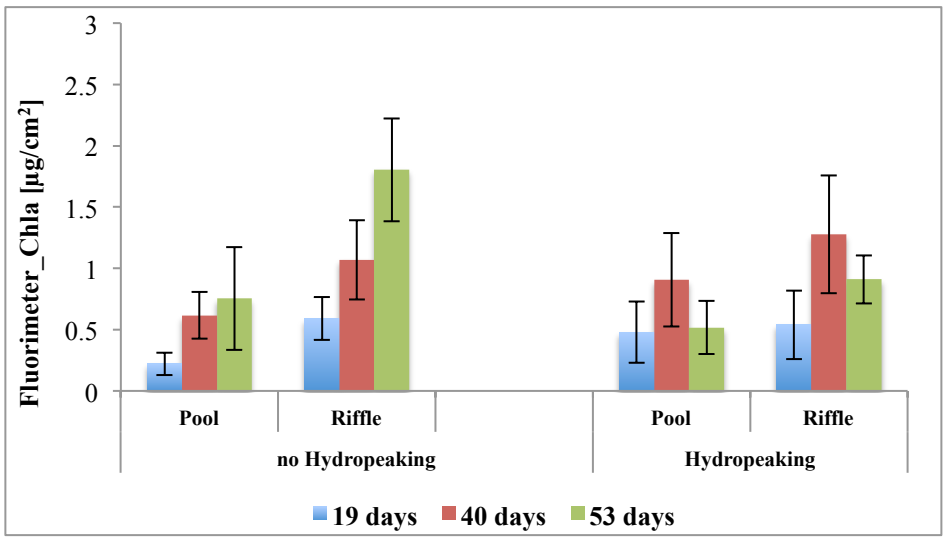

Fig.5 Mean±SD chlorophyll a total measured by fluorimeter from 3 dates of experiment, samples were taken from two different habitats, 6 points from each habitat, Chl a shown in [µg cm2]

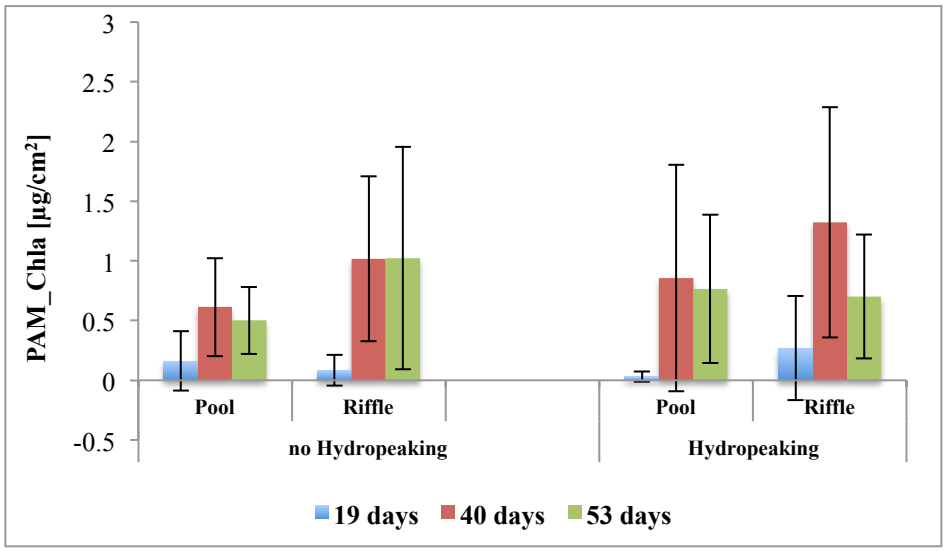

Fig.6 Mean±SD chlorophyll a total measured by Phyto-PAM from 3 dates of experiment, samples were taken from two different habitats, 12 points from each habitat, Chl a shown in [µg cm2]

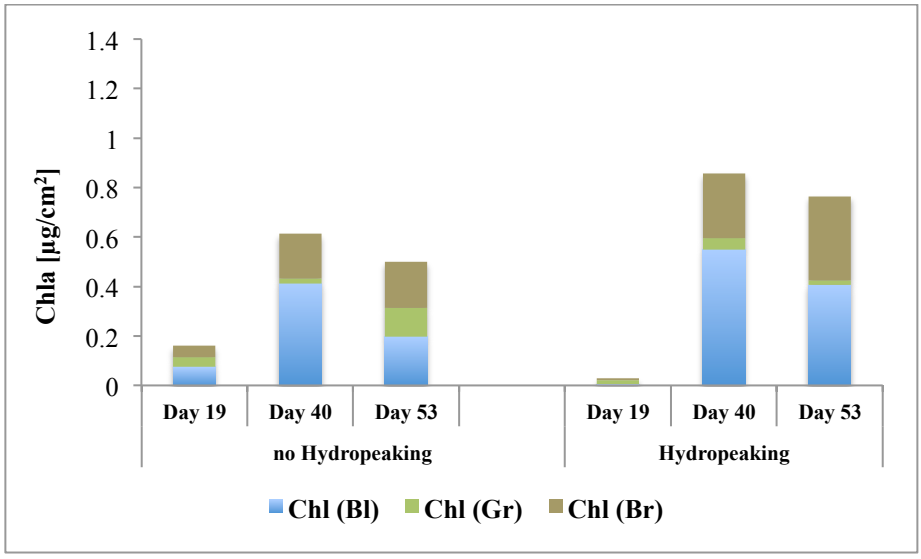

Fig.7 Succession of periphyton community and classification of algal class by PAM measurement from HYTEC experiment at pool habitat

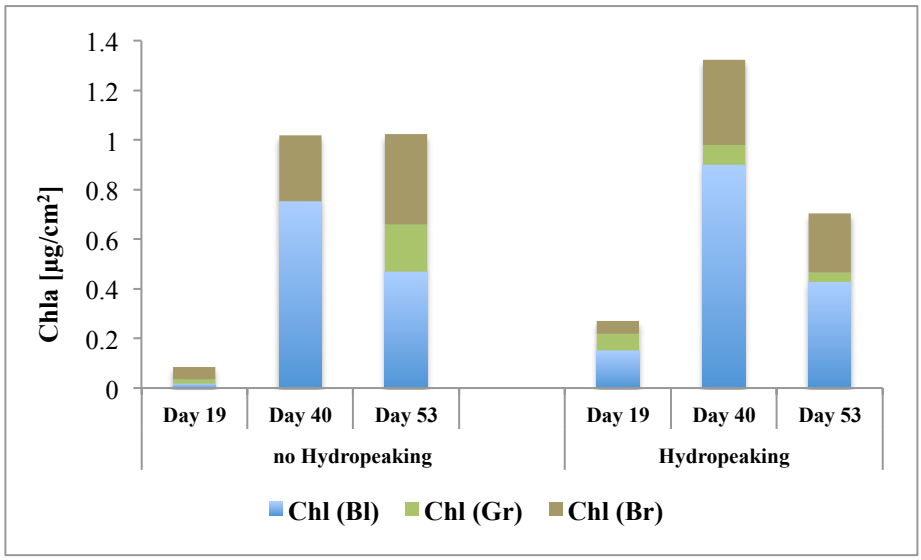

Fig.8 Succession of periphyton community and classification of algal class by PAM measurement from HYTEC experiment at riffle habitat

PAM		19 days			40 days			53 days		
		% (Bl)	% (Gr)	% (Br)	% (Bl)	% (Gr)	% (Br)	% (Bl)	% (Gr)	% (Br)
No Hydropeaking	Pool	46	24	29	67	3	30	39	23	38
	Riffle	22	21	57	74	0	26	46	19	35
Hydropeaking	Pool	17	56	28	64	5	30	53	3	44
	Riffle	56	24	19	68	6	26	61	5	34

Table.9 The percentage of chlorophyll a concentration of three algal groups (Blue-green, Green, Brown) measured with PAM from HYTEC experiment

Discussion

The aim of this thesis was to test if the Phyto-PAM device can be a reliable tool for assessing the benthic algal community, mainly measuring the chlorophyll a concentration, and also estimating the relative contribution of three major algal groups in a fast and non-destructive way. The good linear correlations between the Phyto-PAM measurements and the acetone extraction based fluorimetric measurement of different mixed samples from pure cultures confirmed that the Phyto-PAM delivers reliable results (Fig.1; Fig.2). Similar results showed in a study from Jesus et

al. (2005), which stated the use of non-invasive fluorescence method were used successfully to show the variability in biomass distribution in small scale. As they applied the use of PAM fluorescence in investigating the spatial structure and dynamics of benthic algae in term of centimeter scale, the results showed that PAM measurement could provide insights into the short-term dynamics of microphytobenthos assemblages, particularly, by facilitating the discrimination of biomass changes in situ.

Furthermore, the chlorophyll a content was overestimated. A possible reason can be the influences of high irradiance, this because where the density of radiation incident on the surface that we measured; it could cause the overestimation. The work from Kromkamp et al. (2006) also showed the similar outcome, where they used chlorophyll fluorescence in measuring the photosynthetic properties of the benthic algae, the results showed the overestimation of the true apparent quantum efficiency and the estimated rate of photosynthesis, this error is significant at higher irradiance.

Besides, the ability to compute the pigment contents quickly and did not destroy the samples' cell; PAM could also estimate and classify three main algal groups (green, blue-green, diatom). According to Barranguet et al. (2004) the fluorescence methods could give an accurate representation composition in young biofilm. This statement also supports our finding where thin and young (initial phrase) algal samples showed better correlation (Fig.2; Fig.3) and presented the accurate portions of algal composition comparing to the known cultures samples and other methods (fluorimetry, HPLC) (Table.8).

On the contrary, measurement of stone samples, where the stone surfaces were rather thick and mostly attached with old algae. The poor correlation reaffirmed that PAM measurement could not detect the thick samples or dense algae, from our study; the samples that contain more than 3-4µg/cm^2 could gave the failed results (Fig.4). Other studies Barranguet et al. (2004); Jesus.B et al. (2005); Aberle et al. (2006); Schmitt-Jansen, M. et al. (2007) had similar results, because they also found out that older and thicker biofilms, were too dense and limiting the light to fluorescence-based method and therefore underestimate the biomass. It could also affect the overall pattern of the benthic algae assemblages.

The influence of hydro peaking on periphyton community structure

According to the second hypothesis, the composition of periphyton community would have changed due to hydropeaking events. The difference could be observed clearly on the beginning of the experiment at both pool and riffle habitats, where the majority group totally changed due to the effect of hydropeaking (Fig.7; Fig.8). These show that disturbances create the heterogeneity in

structure, influence the behavior of the organisms and also establish temporal changes in attached algal communities (Ledger et al, 2008; Hobbs et. al, 1992). Particularly, pool (Fig.7) and riffle (Fig.8) habitats showed the different respond to the change and also different range of biomass as they have the different condition, riffle which is the shallow water with higher flow velocity, algal attached on the substrates there tend to get more light and nutrient more than those in pool habitat which is the deeper with lower velocity, the substrates in the riffle are also more uniform than in pool (Allan J.D et al. 2007) and with these differences the assemblages of the benthic algae are accumulated by different population. Moreover, shallow with high velocity stream assembled higher biomass than the area where is deep and low flowing (Wetzel, 1964; Stevenson R.J et al. 1996).

From our finding, it is also referring to the statement from Zanon et al. (2013) said the disturbance frequency affects the ecosystem respond, the more frequent of the disturbance, the lower the possibility to recover and in turn influences the system variability and change the system dynamic. For some species the flow fluctuation can promote its ability to grow by increase the nutrient availability while some of them could not resist and lost their population by sloughing (Peterson et al, 1992; Larned Scott T., 2010). The resistance of benthic algae can also increase by the frequency of the disturbance over time and the environment they exist. According to (Peterson et. al, 1992) the algae that grow in the high shear stress attach more strongly to the substrates and having higher resistance to scour than those who grow in the slow current habitats. In short, the longer the algae propose to the stress/new environment the more they could adapt to the changes but it also depends on other factors like nutrients, light, temperature as well. Moreover, the community structure would change from its initial form and may be develop in total different structure.

Using PAM measurement on assessing the effects of hydropeaking on the succession of a periphyton community

When applied PAM measurement on assessing the changes of periphyton community, the findings is in accordance to other observations. Schmitt-Jansen M. et al. (2007) stated that multi-wavelength PAM fluorometry revealed sufficient patterns of the main benthic algae groups (diatoms, chlorophytes and cyanophytes). PAM also provides the outlook to observe community structure of periphyton and patterns interacted to the effects or changing over time for both biomass and class composition. In particularly, PAM measurement can illustrate the changing of portions of algal assemblages due to the hydropeaking treatment (Fig.7; Fig.8), but the accuracy

of chlorophyll a content was limited. Otherwise, in comparison to the HPLC data, where analyzing based on the biomarker, and the information regards pigment composition and pigment ratios of microalgal groups is stressed in the literature (Brotas et al. 2003). Besides, PAM could also gives better pictures with higher variability on community development as it is able to measure the wide range of samples in a short time (Fig.6). Unlike the extraction based methods (fluorimeter, HPLC) (Fig.5) where only some spots was investigated, as this method could only collect specific samples to measure and to get the overview of the whole community it would consume more time. Therefore, in our studies, PAM measurement could be a better option to use in term of studying the composition changing over time, the measurement based on good references of each algal group, which easily made by cultures to be more accuracy. Furthermore, this could be the best advantage of PAM techniques, where the device could rapidly estimate differences without having to wait for further analyses apart from determination of total algal biomass (Aberle et al. 2006).

Consequently, PAM fluorescence techniques could be the very good instrument in assessing the patterns of biomass variability and composition changing of benthic algae community over time. However, there are some issues that need to be taken into consideration. Most importantly, the algal samples' thickness since the device is only able to measure the thin area where light could go through the samples. Also taking into account that PAM measurement could not determine the accurate values of the chlorophyll a concentration but only the overall pattern of changing over periods of time, at present. As the chlorophyll contents based on the calibration, having the good calibration is essential. Therefore, calibration should also be made for different types of algal samples and as many varied substrates as possible (Aberle et al. 2006). In addition, Phyto-PAM should be used in collaborative with other destructive method where the samples should be more homogenous and measured for whole samples, doing that way, the results should be more precise. Amann R et al. (1998) also pointed out that to study structure and functioning of microphytobenthos communities, combining different methods should be applied. Hence, PAM measurement could become an important device in aquatic benthic study as it is very fast tool to the qualitative and quantitative assessment of periphyton and can be done in situ, also it does not required many other equipment. Even the biomass values were underestimated but the overall patterns are fit for use in studying the community changing especially, in the long-term experiment. Further measurement will develop and redefine the better use, in order to be more accurate on biomass contents and also the algal difference groups' classification.

Conclusion

PAM fluorescence technique is a very useful tool for periphyton community assessment as it provides a good overview of the community structure and changes over time with high variability and quantitative measurement. It is also able to give a fast discrimination of major algal groups in situ and non-destructive cell of samples unlike other methods, see Table.10 for methods comparison. Besides, PAM measurement also provides the ability to observe the photosynthesis activity of the algal community. A restriction may occur when algal growth differing in age or depth, thus the differentiation of samples should be compared and the calibrations should be made base on each type of sample that we measured for the more suitable chlorophyll a contents. Furthermore, in term of operation, PAM technique would be the preferable option, as it is very easy to set up/implement, do not need much time or updates, it is also not very expensive, outstandingly, PAM can be used in situ while other methods could not. Therefore, with the non-invasive and no requirement of time consuming process, PAM has become the reliable method for screening of changes on structural of microalgae community.

Method	Strong points	Weak points
PAM	Quick/no requirement to prepare samples (raw samples can be measured), wide range sample measurements (young biofilm), non-destructive cell, be able to distinguish three algal class in situ, also give more info rather than Chl a value (ETR)	Unable to detect the thick/old biofilm could not handle the large amount of samples. Chl a conc. depends on good calibration
HPLC	Handle amount of samples, in details and get the actual value of each pigmentation that contain in samples	The sum of Chl a cannot be use, analyze data with CHEMTAX depends on ratios (can be inaccuracy), consume more time, sample collecting and storage
Fluorimeter	Easy to measure samples, can measure whole samples without barrier (thickness), give the precise and actual value	Only give the sum of Chl a value of the samples, consume time in preparing and extraction, sample collecting and storage

Method	Cost*	Updates	Setup	Measurement	Time
PAM	Fair	None	Easy	Easy	Fast
HPLC	Most expensive	None	A bit complicated/need to prepare reagent	Relatively easy	Need time to extract samples/ run time (30mins/sample)/peak analyses
Fluorimeter	Cheapest	None	Easy	Easy	6-16hours to extract samples

Table.10 Method comparison, a comparison of the three measurement used in the study

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