

## Innovative approaches for measuring organism stress and behavioural integrity in flume facilities

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## **EXECUTIVE SUMMARY**

HYDRALAB+ aims to improve the usefulness and value of hydraulic laboratory facilities and is developing experimental guidelines that will allow researchers to successfully investigate complex scenarios representative of natural environments in a context of climate change. Within this framework it is often important to incorporate relevant biological elements in physical experiments, including the use of live vegetation. Notwithstanding efforts to maintain their health by careful husbandry, plants typically degenerate when introduced to flume settings. Physiological responses to degenerating health can affect their interactions with the flow so that experimental conditions are not representative of healthy specimens in situ. There is therefore a need to measure and evaluate the health of plants being used in hydraulic facilities, especially since behavioural integrity might be reduced before there are obvious signs of degeneration. Such measurements are not routinely made so there is a need to identify measurement techniques and methodological protocols for assessing vegetation health status in hydraulic laboratories.

This deliverable identifies a technique established in plant physiology and horticulture for monitoring vegetation health status and shows how it can be applied in hydraulic laboratories with minimal impact on organisms. A simple and suitable test among those established in the relevant literature is validated by conducting experiments on freshwater macrophytes. From the relevant literature and the results of experiments reported herein, this deliverable provides an overview of the technique identified and establishes practical guidance on how to properly apply it in hydraulic experiments. The methodological protocol developed can potentially be integrated into established protocols used in ecohydraulics studies as a simple proxy of vegetation health status.



## 1 Introduction

In the context of climate change, physical hydraulic modelling is crucial for forecasting the effects of future climate scenarios on hydraulic infrastructures, hydrological risk, and aquatic environmental systems. To develop a holistic knowledge, experimental models should incorporate the interactions of forcing mechanisms with other components of aquatic environments including biota. Vegetation is an essential component of aquatic ecosystems that affect biological, ecological, chemical, and physical processes (e.g. Bornette and Puijalon, 2011; O'Hare, 2015). Among hydraulic researchers there has been an expanding interest in plants reflected in several recent reviews including: Folkard (2011) on vegetated flows, Nepf (2012) on hydrodynamics of vegetated channels, and Gurnell (2014a) on the role of plants as river engineers. To maximise control over experimental conditions, most laboratory studies have been conducted using plant surrogates (Thomas et al., 2014b), but use of live plants guarantees a more comprehensive representation of natural systems, so there is significant and increasing interest in the use of live specimens in flumes. Notwithstanding good husbandry, plant health is difficult to maintain once the plant is removed from its natural environment. Indeed, exposure to environmental conditions typical of flume facilities may affect plant health by driving specific physiological responses that can, potentially, lead to modifications in plant biomechanical properties and, therefore, the way in which the plant interacts with the flow. A key question when using live plants in flume facilities is, therefore, whether their health has deteriorated to a point where their behaviour is outside the range of natural behavioural variability. For example, in the extreme, the tissues of a dead plant will not have the same biomechanical properties as the tissues of a live specimen and so changes in material buoyancy and/or elasticity are likely to cause plant-flow interactions that are not representative of healthy specimens. Atypical behaviour caused by poor health may develop before a plant is obviously dead, in which case the researcher would be unaware of this problem. It is then possible that observed experimental differences in flow properties caused by plant ill-health mask the effects (or non-effects) of the treatments that an experiment is designed to investigate, potentially undermining the validity of the results obtained. For these reasons, it is desirable that hydraulic facilities have an ability to monitor and evaluate vegetation health status.

With this report we aim to provide hydraulic researchers with a simple and effective tool that can be used to quantitatively assess vegetation health status in laboratory experiments. First, we identified a suitable technique – chlorophyll fluorescence analysis – and a test, the  $F_{\nu}/F_{m}$  test, that are established in the botanical literature. We then applied the technique in a range of situations to test its effectiveness and develop relevant guidelines for use by hydraulic researchers. Our applications focused on freshwater macrophytes, a type of vegetation that has recently gained interest in the hydraulic community, because they are generally very flexible plants and hence are expected to show a stronger biomechanical and hydrodynamical response to hydraulic stress. Freshwater macrophytes were also easier to source and handle. O'Hare et al. (2018) have pointed out that research on freshwater macrophytes is expected to become increasingly important with the effects of climate change on freshwater ecosystems.

Section 2 of the report provides a review of work in relevant areas, including an overview of live-vegetation use in flumes, a summary of established approaches to husbandry, consideration of the laboratory conditions expected to affect vegetation health, and the approaches/techniques currently available for measuring vegetation health status. Section 3 is dedicated to the selected



technique, chlorophyll fluorescence analysis. It provides an overview of the relevant biochemical processes, a comprehensive description of the technique, and information on the instruments available. Section 4 reports on a protocol for measuring vegetation health status in hydraulic applications and remaining practical issues. It also contains results of methodological experiments with which we refined the protocol and a step by step description of how to apply it. In section 5 we describe a few examples of applications of chlorophyll fluorescence analysis in laboratory experiments. Section 6 summarises the most important methodological findings of the study and outlines a short roadmap for this research area.

## 2 CONTEXT

## 2.1 Use of live vegetation in flume facilities

A quick search of the hydraulic literature reveals that live vegetation has been used in many laboratory studies since the mid-20<sup>th</sup> century. Studies using live vegetation in flume facilities encompass a diverse range of purposes and topics. At a first approximation and considering vegetation relevant to hydraulic research, vegetation is here classified into six categories: seagrass, seaweeds, saltmarsh plants, riparian vegetation (e.g. trees), grasses and freshwater macrophytes. The large number of plant species that grow below, floating on, or emerge up through the water surface, that are large enough to see with the naked eye and that are not algae are referred to as macrophytes (Cushing and Allan, 2001). These plant species can be found in freshwater or marine environments and include seagrasses.

Seagrasses. Compared to other plants, the use of live seagrass in flume experiments is relatively limited. While not explicitly mentioned in literature, there are two likely reasons for this. First, keeping seagrass alive and healthy in a flume is difficult and requires salt water, which is problematic in many facilities. Second, many researchers have been content to represent seagrass using surrogates. The use of live seagrass was more common in earlier studies of plant-flow interactions when knowledge of suitable surrogates was limited (e.g. Fonseca and Fisher, 1986; Gambi et al., 1990), but a number of contemporary studies do still make use of live specimens. Typical reasons for using live seagrass in hydraulic experiments are to study the effects of hydro- and morpho-dynamics on biological processes such as nutrient uptake (e.g. Morris et al., 2008) photosynthesis (Fonseca and Kenworthy, 1987; Koch, 1994) and response to stress (Cabaço and Santos, 2007; Biber et al., 2009), for which live plants are essential. Also, studies of sedimentation/erosion processes with natural substrates have used live plants (Ganthy et al., 2015; Wilkie et al., 2012). Others have used live seagrass because their interest was in the effect of a particular species rather than general plant traits, which are easier to mimic, on flow dynamics (Peralta et al., 2008). Somewhat outside the scope of hydraulics is the field of interactions with other organisms (e.g. González-Ortiz et al., 2014). Zostera noltei (dwarf eelgrass) is the most common species used in flume studies. Since this species grows in the intertidal zone it is relatively easy to get from the field intact, and its small size make it fit in laboratory flumes. Moreover, its tolerance to intertidal conditions (heat, desiccation) means it can be kept alive easier than more delicate species.

Seaweeds. A large number of investigations have been carried out with live seaweeds in flume environments to deepen the understanding of reconfiguration and exchange processes at the scale of a single organism (e.g. Gaylord and Denny, 1997; Boller and Carrington, 2006; Huang et al., 2011;



Demes et al., 2013; Starko et al., 2015), as well as the effect of environmental stresses on the seaweed's physiology and functioning (e.g. Gerard, 1987; Koehl and Alberte, 1988; Davison and Pearson, 1996; Koehl et al., 2008). Huang et al. (2011) investigated the motion of seaweed blades in unidirectional current in a laboratory flume and included a good description of the handling of kelp blades, but no monitoring of the potential effects of stress. This work echoed the pioneering investigations of Koehl and Alberte (1988), who characterized the functional consequences of blade morphologies in a small flume by monitoring the shear velocities at the blade surfaces, the drag forces and the consequences on photosynthetic rates. During the Hydralab IV project, research activities investigated the feasibility to replicate the hydraulics of a vegetated tidal inlet in a flume environment with and without live seaweeds (Thomas et al., 2014a). In general, environmental stress on seaweeds is well documented in their natural environment, which provides a lot of information for a potential transposition to flume environments. Due to their position on the shore, intertidal seaweeds have developed the capacity to resists changes in salinity, temperature and nutrient conditions (e.g. Davison and Pearson, 1996). For intertidal seaweeds, air exposure may induce a desiccation stress, and the photosynthesis capacity may be recovered during rehydration only in higher intertidal species (Flores-Molina et al., 2014). Experimental studies on large patches of live seaweeds are not common, probably because large seaweed canopies are present in deep environments, while shallower patches tend to cover smaller spatial scales close to the shoreline (Denny and Gaylord, 2002).



Figure 1 (a) Excavation of saltmarsh blocks (2013). (b) Nurturing plants at the NIOZ (Yerseke, Netherlands, 2018). (c) Watering of saltmarsh blocks at the site of the GWK (Hannover, Germany, 2013). (d) View of the experimental setup of the RESIST experiment from inside the large wave flume (Hannover, Germany, 2013, adapted from "The RESIST team").



Saltmarsh plants. Saltmarshes are meadows of different salt-tolerant plants that are mostly located in the upper coastal intertidal zone where they experience regular inundation. Besides their role as important ecosystems saltmarshes have been recognized for their ability to protect coastal regions by reducing wave energy and soil erosion (e.g. Barbier et al., 2011). Uncertainties surrounding the interactions between these plants, the soil and (extreme) hydrodynamic forcing have motivated a transnational team to conduct true-scale experiments within the Hydralab IV project. These experiments were conducted using live saltmarshes in the large wave flume (GWK) in Hannover (Figure 1). Several blocks of saltmarsh turf were excavated from a natural saltmarsh on the mainland coast of the German Wadden Sea (Möller et al., 2014). The mixed canopies consisted of Elymus athericus, Puccinellia maritima, and Atriplex prostrate. These blocks were then stored at the site of the GWK for one year where they also overwintered. For the cold season, a straw insulation was installed and the plants were watered regularly using saltwater during warmer periods. In 2013 the marsh blocks were transported into the flume to carry out experiments which investigated the potential of a 180 m<sup>2</sup> test section of saltmarsh turf to dissipate wave energy under storm conditions. The same tests were run using a mowed saltmarsh to compare the wave attenuation potential of a vegetated state with a bare state (Möller et al., 2014; Spencer et al., 2016). A follow-up experiment was conducted in 2018 focusing on the sediment stabilization potential of salt marshes (RESIST – "Response of Ecologically-mediated Shallow Intertidal Shore Transitions to extreme hydrodynamic forcing") and the negative impact of sediment dynamics on young growing plants (loss of vegetation) to improve understanding of how to manage vegetated tidal flats under future climate scenarios. Maza et al. (2015) and Lara et al. (2016) investigated wave attenuation caused by salt marshes species P. maritima and Spartina anglica under a range of flow conditions and considering both currents and waves.

Riparian plants. Vegetation typical of riparian areas and floodplains, such as trees and crops of diverse types, have been used in flume experiments for investigating flow resistance, flow characteristics above a canopy, and the drag force experienced by individual organisms. Live willows were used by Felkel (1960) and by Armanini et al. (2005) for studying flow resistance. Armanini et al. (2005) achieved this by direct measurements of the drag force experienced by willows located in a 150 m long and 2 m wide and deep flume under partially and fully submerged conditions. Rahmeyer et al. (1996) investigated a total of 20 natural plant species in two different flumes with varying plant density. Flow velocity and depth, plant density, dimensions and types were varied in the experiments. Using a drag force measurement device, the drag force exerted on a vegetation element was measured during each experiment. Diverse types of crops were used in other studies. For example, Haber (1982) conducted flume experiments with different types of flexible vegetation (wheat, rye, barley, rape, oat and grass) on both cohesive and non-cohesive substrates. Järvelä (2005) used live wheat plants grown in metal boxes to study the mean vertical profiles and turbulence characteristics above a patch of flexible vegetation. Wilson et al. (2008) investigated the impact of foliage on the drag force experienced by branches of pine (Pinus sylvestris) and stipes of ivy (Glechoma hederacea).

Grasses. Smaller types of vegetation have been used in flume experiments for studying flow resistance and responses of the flow to natural flexible elements (e.g. Carollo et al., 2002; von Liebermann et al., 2005) and for investigating the morpho-dynamics of fluvial systems (e.g. Gran and Paola, 2001; Tal and Paola, 2007; Bertoldi et al., 2015) and deltas (e.g. Piliouras et al., 2017). The latter work on morpho-dynamics has frequently used small plants (like alfalfa grown from seed) to simulate the role of larger plants (like floodplain trees) and the cohesive effects of their roots in



analogue or landscape-scale physical models. Using small plants that rapidly germinate from seeds has helped to establish the fundamental role that vegetation can have on channel stability and planform behaviour in fluvial systems (Gurnell, 2014b). Research at St. Anthony Falls Laboratory (Gran and Paola, 2001) is the first recorded use of germinating small plants in landscape-scale physical models. In these experiments, alfalfa (Medicago sativa) were seeded on an existing scale model of a braided river system. Pre-soaked and air-dried seeds were dispersed uniformly at half flow conditions over the braided river system at various densities. Under damp or very low flow conditions the seeds germinated for 10 - 14 days. This method proved to be successful and subsequent research applied a similar methodology with slight adaptations (e.g. Tal and Paola, 2007; Braudrick et al., 2009; van Dijk et al., 2013; Bertoldi et al., 2015). In several of these studies a significantly shorter growth duration has been used, which yields slightly shorter stems and roots. Furthermore, van Dijk et al. (2013) compared uniform seeding over the floodplain with a method that added seeds to the inlet to allow natural spread by flow in meandering systems. In addition to alfalfa, other species of small germinating plants have also been used including common oats (Avena sativa) (Perona et al. 2014) and garden rocket (Eruca sativa) (van de Lageweg et al. 2010). Experiments by Kleinhans et al. (2014) explored the impact different species have on bank erosion rates to understand how these small plants impact on morpho-dynamic processes.

Freshwater macrophytes. In recent years, flume experiments have been undertaken to study the hydrodynamics of freshwater macrophytes and the drag forces they experience (e.g. Biehle et al., 1998; Sand-Jensen, 2003; Schutten et al., 2004; Siniscalchi and Nikora, 2013). Most of these experiments have been conducted at a small scale using individual stems or plants. A wide range of species have been used in flume experiments, the most common being *Callitriche* spp., *Elodea* spp., Myriophyllum spp., Potamogeton spp., and Ranunculus spp. Schutten et al. (2000, 2004) conducted experiments with multiple freshwater macrophyte species to measure the drag force acting on them caused by currents (Schutten and Davy, 2000) and waves with a fixed period and wavelength (Schutten et al., 2004). Plants were collected from shallow lakes in the UK and experiments were conducted on individual shoots cut from the plants. Sand-Jensen (2003, 2008) conducted similar experiments measuring the drag force experienced by shoots of several freshwater macrophyte species collected from a stream during summer. Both Schutten and Davy (2000) and Sand-Jensen (2008) aimed to find a relationship between the drag force experienced by a freshwater macrophyte and its biomass. Siniscalchi and Nikora (2012, 2013) tested individual stems of several species in fully submerged conditions and across a range of flow scenarios. They investigated the dynamic reconfiguration (i.e. motion) and drag forces experienced by freshwater macrophytes and the way in which they interacted with the turbulent flow. While most studies have been limited to physical aspects, Bal et al. (2011) and Asaeda et al. (2017) provide two examples that also included biological observations. Bal et al. (2011) investigated the trade-off between hydrodynamic forcing and the photosynthetic capacity of freshwater macrophytes by measuring the drag force experienced by shoots and their relative photosynthetic surface area. The relative photosynthetic surface area was used a proxy for photosynthetic capacity even though it underestimates the real surface area exposed to light. Asaeda et al. (2017) studied the physiological stress of three freshwater macrophyte species associated with exposure to a range of flow velocities and turbulence levels for eight weeks. Plants were collected from a river and acclimated in aguaria for more than a month prior to the experiments. Plant stress was estimated by measuring the concentration of Reactive Oxygen Species (ROS) in plant tissues and other biological parameters.



The use of live freshwater macrophytes at larger spatial scales (e.g. patch, canopy) in flume facilities is rare. Stephan and Gutknecht (2002) investigated roughness caused by freshwater macrophytes and their effects on mean velocity profile by testing three species under submerged conditions. Experiments were conducted in a 17.5 m long and 1 m wide flume with a 3 m long patch of plants that covered the full width of the flume. Plew et al. (2008) conducted experiments in an outdoor artificial flume (14 m long and 0.75 m wide) with 10 m long canopies made of stems of *Lagarosiphon major* collected from a nearby river. They investigated how canopy density influenced both mean and unsteady forces acting on freshwater macrophytes within a patch. A range of canopy densities and flow rates were used, and the drag force was measured on a single stem within the canopy.

#### 2.2 HUSBANDRY PRACTICES FOR VEGETATION IN FLUME FACILITIES

Vegetation can be sourced either by growing it in dedicated facilities (e.g. Tal and Paola, 2010; Lara et al. 2016) or by collecting it (e.g. Sand-Jensen, 2003; Siniscalchi and Nikora, 2012), the latter method being the most common. The use of live plants in flumes has not been coupled with an extensive effort to develop guidelines for plant husbandry. Although general issues are addressed in Frostick et al. (2011, 2014) and Lara et

al. (2016), there is a need to develop more specific guidelines that would help to establish a set of standard practices. A few principles of husbandry for vegetation in flume facilities can be deduced from relevant literature:

- Vegetation should be selected depending on the size of facility, the characteristics of the type of vegetation to be studied and the focus of the study. These considerations include choosing plant species and scale bearing in mind that plants show a great variability in morphological and biomechanical characteristics both between different species and within a species (e.g. Miler et al., 2014).
- Environmental conditions during storage should be representative of the vegetation's natural environment. Also, when choosing the experimental period, proper attention should be given to the plant's life cycle because seasonality can have a significant impact on plant biomechanical properties (e.g. Miler et al., 2014).
- Depending on the type of vegetation and focus of the experiments, vegetation can be potted
  or rooted (i.e. moved into the facility with the soil in which it has grown) or can be fixed to
  the flume bed by means of artificial objects. It is also important to bear in mind that
  environmental conditions in the storage facility or natural environment and conditions in the
  flume facility may differ significantly.
- Proper considerations should be given to the temporal scale of the experiments and
  environmental conditions of the facility and how these relate to vegetation performance and
  health. For example, experiments may need to be conducted after vegetation has had
  sufficient time to acclimate to the conditions (e.g. days or weeks), or immediately after
  vegetation has been located in the facility if conditions are expected to accelerate plant
  deterioration.

Many contemporary studies provide information on the environmental conditions that vegetation is exposed to during experiments and explicitly consider essential factors such as light and nutrient availability, aeration, and water quality. However, each study is characterised by different conditions that are driven mainly by technical constraints rather than by vegetation healthcare or botanical



expertise. These aspects have been overlooked until recently and the lack of standard practice has the potential to impact on vegetation and its performance in experiments.

# 2.3 IMPACT OF LABORATORY CONDITIONS ON VEGETATION HEALTH STATUS AND PERFORMANCE

As established by a number of studies, environmental conditions can affect plant health status and growth (e.g. Imamoto et al., 2007; Asaeda and Rashid, 2017). Although most researchers use live plants within a few days of collection, even a short-term exposure to stressful conditions can affect plant health (e.g. Hanelt et al., 2006; Hussner et al., 2010). Bornette and Puijalon (2011) reviewed the most important abiotic factors for freshwater macrophytes, excluding contaminants, and the ways that they affect plant life. They identified five factors: light, temperature, nutrient availability, substrate characteristics, and water movement. Each factor can represent a disturbance for macrophytes and all are relevant, to some extent or another, in laboratory settings. The precise importance of each of these factors will vary with the species and the length of time that plants are deployed.

- Light is a key concern because it powers photosynthesis and aquatic plants can be significantly stressed by low or high irradiance levels (e.g. Hanelt et al., 2006; Hussner et al., 2010). Even so, most flume studies with vegetation fail to report information on the light conditions. There are some exceptions (e.g. Siniscalchi and Nikora, 2012), but they tend to provide information about lighting equipment or total light irradiance, which does not allow calculation of relevant quantities, such as the light irradiance that is available to plants for photosynthesis.
- The effects of temperature on aquatic plants have been explored with conflicting results (e.g. Olesen and Madsen, 2000; Pilon and Santamaría, 2001; Malheiro et al., 2013). It is likely that freshwater macrophytes have different acclimation ability depending on the niche to which they belong (Bornette and Puijalon, 2011).
- Nutrient availability is another key factor for plants. Various ecophysiological studies report
  the significant impact of nutrient availability on macrophyte growth and physiology (e.g.
  Madsen and Cedergreen, 2002; Dülger and Hussner, 2017). In the case of freshwater
  macrophytes, nutrients (mainly carbon, nitrogen, and phosphorus) can be absorbed from
  either the water column or the substrate (Bornette and Puijalon, 2011).
- In streams, characteristics of the substrate are important for defining what species anchor to the substrate. A few freshwater macrophytes can grow on rocky substrate, while most species grow on fine cohesive sediments by means of shallow roots (Bornette and Puijalon, 2011). As reported by Madsen and Cedergreen (2002) for four submerged macrophyte species, growth of freshwater macrophytes in nutrient-rich water is not limited by available substrate. Therefore, if adequate water nutrient content is provided to plants, the substrate chemistry is probably less important than its physical characteristics. Physical characteristics of the substrate are well described in geomorphological studies (e.g. Tal and Paola, 2010; Bertoldi et al., 2015), while they are not considered in hydrodynamic studies (e.g. Bal et al., 2011; Siniscalchi and Nikora, 2012).
- The characteristics of the flow in which plants are immersed have complex effects on their health (Bornette and Puijalon, 2011). Some pioneering studies on the effects of flow characteristics on plant health status have been conducted by Asaeda and Rashid (2017) and

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Asaeda et al. (2017), who reported that high levels of turbulence are stressful for some freshwater macrophyte species. Flow characteristics are well documented in studies focusing on plant hydrodynamics (e.g. Siniscalchi and Nikora, 2012).

Environmental conditions that influence vegetation health status and growth, are also expected to affect plant biomechanical properties as stress and ill-health produce a physiological response. In turn, biomechanical properties (e.g. buoyancy and elasticity) may change sufficiently to influence the way in which plants interact with the flow and, consequently, compromise the results of laboratory experiments. With a very recent notable exception (Asaeda and Rashid, 2017), there are no examples in the ecohydraulics literature of how to quantitatively assess the health status of plants used in flume facilities.

## 2.4 APPROACHES TO MEASURING VEGETATION HEALTH STATUS

Most researchers are likely to assess plant health before an experiment by performing a visual check, but plants may be stressed without any outward signs of deterioration. Plant growth rate can be monitored relatively easily as a proxy for health, but it is relevant only in experiments with long temporal scales (e.g. Madsen and Cedergreen, 2002; Asaeda and Rashid, 2017). To cover all experimental conditions, it is, therefore, necessary to focus attention on aspects of plant function that convey information of plant health and occur at short temporal scales. The two most suitable candidates are plant metabolic activity and plant signalling networks. The former deals with the set of biochemical reactions that provide energy to a plant, while the latter describes the complex biochemical responses (or signals) of cells within a plant.

Plant signalling networks comprise a large set of processes that control RNA transcription and the synthesis of metabolites and proteins (Cramer et al., 2011). These processes are the most responsive to stresses and they occur on very short time scales. Reactive Oxygen Species (ROS) such as hydrogen peroxide have been studied for decades because their concentration is a good indicator of the stress experienced by a plant due to biotic (e.g. Mehdy, 1994) or abiotic factors (e.g. Jeffers et al., 2007; Asaeda and Rashid, 2017). Techniques to monitor RNA or measure concentration of metabolites and proteins, however, present some substantial drawbacks for ecohydraulics applications in that they are destructive – small portions of plants must be extracted and analysed – and the methods require chemical analyses that go beyond the standard expertise of and equipment available in most experimental hydraulic facilities. Moreover, results are species dependent so different benchmark values for defining 'healthy' plants are identified for different species (Cramer et al., 2011).

Plant metabolic activity includes all processes through which plants extract energy from the environment such as gas exchange and photosynthesis. These processes are not as responsive as plant signalling networks to stresses and tend to indicate more substantial stresses only (Cramer et al., 2011). The advantage of processes at this level is that they can be monitored with non-destructive techniques that do not require use of chemical analysis. Monitoring gas exchange is probably the most accurate way of assessing plant metabolic activity as it provides direct measurements of oxygen and carbon dioxide fluxes between a plant and the air surrounding it (e.g. Koch, 1994). However, the technique is impracticable in flume facilities because it requires the use of a closed microcosm. Alternatively, photosynthetic activity can be monitored using chlorophyll fluorescence analysis, a technique that measures the photosynthetic performance of leaves and other tissues containing chlorophyll in vivo. From the information obtained, it is possible to estimate



CO<sub>2</sub> assimilation (Baker, 2008). Chlorophyll fluorescence analysis and its numerous applications have been reviewed in the past two decades by Maxwell and Johnson (2000), Baker and Rosenqvist (2004), Baker (2008) and Murchie and Lawson (2013). This technique appears to be the most promising for ecohydraulics applications because it is well established, can be used to conduct a range of tests that provide information about different aspects of photosynthetic performance, and is characterised by indicators that are species-independent (Maxwell and Johnson, 2000; Baker, 2008; Murchie and Lawson, 2013).

## CHLOROPHYLL FLUORESCENCE ANALYSIS

#### 3.1 **OVERVIEW**

Chlorophyll fluorescence analysis is a non-invasive and non-destructive technique. It has been widely used for decades for studying photosynthesis of terrestrial plants (e.g. Maxwell and Johnson, 2000; Murchie and Lawson, 2013) and phytoplankton (e.g. Suggett et al., 2010), but it is relatively novel in the study of aquatic plants. Chlorophyll fluorescence analysis has been used in crop improvement research because it is very sensitive to changes in photosynthesis and measurements are very accurate (e.g. Baker and Rosenqvist, 2004; Kalaji et al., 2014).

#### 3.1.1 Photosynthesis and chlorophyll

Photosynthesis is the result of several complex biochemical processes with which autotroph organisms, such as higher plants and algae, convert light energy into chemical energy that is used to power all activities within the organism. In plants, photosynthetic activity leads to the production of carbohydrates, molecules with high energy content, that are broken down to fuel activities such as plant growth or stored for later use (e.g. in winter). From an energetic point of view, the fundamental part of photosynthesis occurs in a functional unit, referred to as photosynthetic unit, that consists of a photosystem and a light-harvesting complex, also referred to as antenna pigment (Kirk, 1994; Murchie and Lawson, 2013). Depending on the type of reactions they perform, the set of photosynthetic functional components, or reaction centres, are identified as Photosystem I (PSI) or Photosystem II (PSII) (Kirk, 1994). Light energy is absorbed by light-harvesting complexes in the form of photons, which are then used to split water molecules and extract electrons to be used for photochemical reactions that occur in reaction centres (e.g. Murchie and Lawson, 2013). The reaction centres, therefore, allow the conversion of light energy into chemical energy for use by the organism (Kirk, 1994; Falkowski and Raven, 2013). Reaction centres cannot always accept new electrons, as they can 'carry' one electron at a time. Therefore, reaction centres are said to be 'closed' when they cannot accept new electrons, whereas they are referred to as 'open' when they can accept electrons (e.g. Kirk, 1994; Maxwell and Johnson, 2000; Baker, 2008; Falkowski and Raven, 2013).

Plant photosynthesis is dependent on green pigments called chlorophyll. Due to their nature, chlorophyll pigments predominantly use light within a range of wavelengths between 400 and 700 nm (e.g. Falkowski and Raven, 2013). Irradiance in the 400-700 nm waveband is therefore considered to be the component of total spectral irradiance that is photosynthetically active and is referred to as Photosynthetically Active Radiation (PAR) or Photosynthetic Photon Flux Density (PPFD) (e.g. Suggett et al., 2010). The unit of measurement used for PAR is mol quanta m<sup>-2</sup> s<sup>-1</sup>. Within this waveband (and adjacent wavebands), pigments absorb photons with different efficiency



depending on their specific wavelength (McCree, 1971). The most common pigment, chlorophyll *a* (chl-*a*), has two main absorption bands (Figure 2): a blue (or Soret) band at about 450 nm, and a red (or Q) band at about 650 nm (e.g. Falkowski and Raven, 2013). PAR meters (or quantum meters) are available that provide direct measurement of PAR (i.e. from the 400-700 nm waveband). For a full account of photosynthesis in aquatic environments we refer readers to excellent books covering this topic (Kirk, 1994; Falkowski and Raven, 2013).

#### 3.1.2 Chlorophyll fluorescence

Fluorescence is the term used to describe the re-emission of energy in the form of a photon that occurs when an electron undergoes relaxation (Kirk, 1994). In the case of chlorophyll pigments, if a photon is absorbed and not dissipated as heat or used for photochemistry, it is re-emitted with an emission peak shifted at ~685 nm (Suggett et al., 2010). This red-shifted re-emission constitutes chlorophyll fluorescence and represents a small portion of the absorbed light (Figure 2). The intensity of chlorophyll fluorescence emitted depends on factors such as the light conditions and the status of the reaction centres within a sample. Emissions are at minimum when reaction centres are 'open' because the chance of photons being used for photochemistry is maximised. Conversely, emission of fluorescence is maximised when reaction centres are 'closed'.

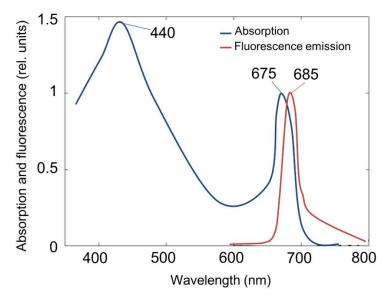


Figure 2 Spectra of in vivo light absorption and of fluorescence emission by chlorophyll a (adapted from Suggett et al., 2010).

According to a simple model introduced by Butler (1978), photons absorbed by light-harvesting complexes can be either: (i) used to power photosynthetic processes; (ii) dissipated as heat; or (iii) re-emitted as chlorophyll fluorescence (Figure 3). Since these three processes are in competition with each other, measurements of chlorophyll fluorescence provide indirect information on heat dissipation and photochemistry (e.g. Baker, 2008). The fluorescence signal is therefore reduced by photochemical and non-photochemical processes occurring in the leaves that compete with chlorophyll fluorescence, this reduction is referred to as quenching (e.g. Maxwell and Lawson, 2000). Photochemical quenching refers to the fraction of PSII centres that are 'open' and capable of extracting electrons (Baker, 2008). Non-photochemical quenching refers to the effect of several processes on PSII efficiency (mainly heat, for more details see Baker, 2008; Murchie and Lawson,



2013). Even though two photosystems exist, because the contribution of PSI to the chlorophyll fluorescence signal is very low compared to that of PSII at wavelengths within the PAR waveband, it is commonly assumed that chlorophyll fluorescence is emanated by PSII only (e.g. Baker, 2008; Murchie and Lawson, 2013). Furthermore, since PSII is the part of the photosynthetic apparatus most sensitive to stress, its status is the most appropriate indicator of plant stress (Maxwell and Johnson, 2000). From measurements of chlorophyll fluorescence, it is possible to estimate the quantum efficiency of PSII, the flow of electrons through PSII which provides the overall rate of photosynthesis (Maxwell and Johnson, 2000), and the chlorophyll content of leaves (Kalaji et al., 2014).

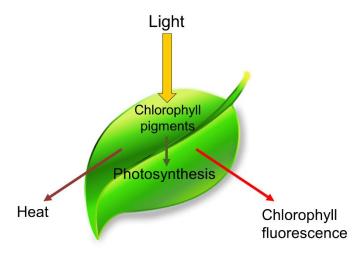


Figure 3 Sketch reproducing the fate of photons absorbed by chlorophyll pigments according to the model proposed by Butler (1978).

Photosynthetic processes have a temperature optimum that depends on several factors (irradiance level, CO2 concentration, plant species and growth pattern, Berry and Björkman, 1980). They also follow diurnal cycles, which means that plant physiological information collected with chlorophyll fluorescence analysis can be masked by variations associated with these cycles (Belshe et al., 2007). Chlorophyll fluorescence can also be very patchy in stressed leaves, therefore intra-leaf and interleaf spatial heterogeneity should be taken into account (use of imaging PAM fluorometer resolves this issue, Baker, 2008). Also, it is worth noting that information extracted with chlorophyll fluorescence analysis may not be representative of the whole organism (e.g. Murchie and Lawson, 2013). Even though chlorophyll fluorescence is not a generic 'plant stress detector' (Murchie and Lawson, 2013); that is, it cannot be used to identify the stresses to which a plant is exposed, it is a powerful tool for comparing the health of plants belonging to a homogenous sample (Baker, 2008), and for monitoring changes in a plant health through time, acclimation to different environmental conditions and tolerance to stresses (e.g. Maxwell and Johnson, 2000).

#### 3.2 DESCRIPTION OF THE TECHNIQUE

#### 3.2.1 Instrumentation

Instruments used for measuring the chlorophyll fluorescence signal emitted by photosynthetic tissues or organisms are referred to as 'chlorophyll fluorometers'. In recent years there has been a consistent increase in the number of user-friendly chlorophyll fluorometers on the market, which has driven an extensive use of chlorophyll fluorescence techniques in plant ecophysiology (Maxwell



and Johnson, 2000; Murchie and Lawson, 2013). Some instruments have been developed for assessing photosynthetic activity of very specific organisms or for use in particular conditions. For example, chlorophyll fluorometers that can analyse photosynthesis within single cells (i.e. with a microscopic sensor), are equipped with multiple sensors, or can analyse samples of water for analysis of phytoplankton are available. Here we focus on instruments that can be used to analyse higher plants in a wide spectrum of applications: non-modulated fluorometers, Pulse Amplitude Modulated (PAM) fluorometers, and imaging PAM fluorometers. Non-modulated fluorometers were the first fluorometers introduced and are used in dark conditions so that the fluorescence they measure is not biased by any background illumination. This limitation was overcome by the development of PAM fluorometers, in which light source and detector are synchronised and modulated at high frequency (e.g. Maxwell and Johnson, 2000). Most instruments available nowadays are PAM fluorometers, so we only consider them here. A particular subset of PAM fluorometers are the imaging PAM fluorometers that allow measurements of chlorophyll fluorescence from whole leaves or multiple samples (e.g. Baker and Rosenqvist, 2004). This type of fluorometers is extremely useful for assessing the heterogeneity across leaves.

Fluorometers are equipped with two different light sources that deliver actinic light and measuring light. The light emitting diode (LED) providing actinic light is usually white and is used to both expose the sample to certain light conditions (for example used in rapid light curves) and to emit high frequency pulses of saturating light (or saturating pulses) that are used to obtain measurements of maximum chlorophyll fluorescence. The measuring light can be blue or red and it is used to measure fluorescence emitted by chlorophyll within a sample. Actinic light has a maximum intensity of a few thousand µmol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup> whereas the measuring light must have a low intensity (e.g. 0.1 µmol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup>) so that it does not drive photosynthesis. Some fluorometers also make use of a far red (FR) low intensity light that is used to obtain more accurate measurements of minimum chlorophyll fluorescence. Fluorometers are often equipped with a fibre optic cable to transmit the light from the sensor/device to a leaf and vice versa. Thus, fluorescence measurements taken by an instrument refer to the sampling area in front of the end of the fibre optic cable. The size of the sampling area is assumed to be equal to the fibre optic cross-sectional area.

A fundamental accessory for any chlorophyll fluorometer is the leaf clip (also called leaf holder), with which a leaf can be secured to the end of the sensor's fibre optic cable, guaranteeing a consistent distance between sensor and sample. Leaf clips with different designs are available, they can often be used for dark-adaptation and can be equipped with PAR and temperature sensors. Cuvettes are also available to measure chlorophyll fluorescence and respiration of a small sample such as an individual leaf. When neither fluorometer nor leaf clip are equipped with temperature and PAR sensor, it is recommended that additional instrumentation to measure them is used. A PAR meter is particularly useful because photosynthetic performance of leaves varies depending on the light irradiance to which they are exposed and adapted (e.g. Baker, 2008; Murchie and Lawson, 2013).

#### 3.2.2 Theoretical background and definitions

According to the model introduced by Butler (1978), chlorophyll fluorescence competes with heat loss and photochemistry for the fate of photons absorbed by light-harvesting pigments (Figure 3). Once a photosystem has extracted energy from a photon (i.e. it has gone into an electronically excited state), it is considered to be 'closed' – it cannot extract energy from other photons – until the excitation energy is transferred to a quinone acceptor ( $Q_A$ ) in the form of an electron. From  $Q_A$  the electron is then transferred to a second quinone acceptor ( $Q_B$ ) and, from there, transferred to

other proteins to power photochemical reactions. As soon as one electron is accepted by  $Q_A$ , the photosystem is 'open' again and can extract a new electron. It follows that quinone acceptors  $Q_A$  and  $Q_B$  become fully oxidized (i.e. with no electrons) soon after photosystems stop extracting electrons, while they are fully reduced when photosystems harvest photons and transfer electrons to the quinone acceptors (e.g. Baker, 2008). A hysteresis occurs between these processes, the status of quinone acceptors adapting to that of photosystems. These variations in the status of the photosynthetic apparatus affect photochemical processes which, in turn, impact on the intensity of chlorophyll fluorescence. In order to describe how we can assess the status of PSII by monitoring chlorophyll fluorescence, in Figure 4 we display an idealized trace of fluorescence obtained using a PAM fluorometer on a dark-adapted leaf. A dark-adapted leaf has been kept in the absence of significant PAR, so that it does not photosynthesise, for enough time so that no quenching is present and all PSII centres are open.

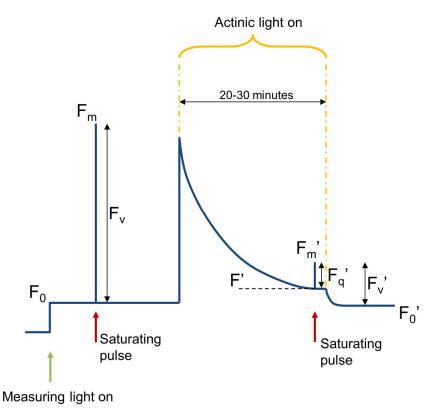


Figure 4 Idealized chlorophyll fluorescence trace of an experiment to perform quenching analysis using a PAM fluorometer on an initially dark-adapted leaf (adapted from Murchie and Lawson, 2013). Note that, as explained in the text, measuring light does not drive photosynthesis, actinic light drives photosynthesis but does not fully saturate the photosystems, pulses drive photosynthesis by saturating photosystems. In periods in which actinic light is off no photosynthesis occurs.

Minimal fluorescence ( $F_0$ ) is measured from a dark-adapted leaf using a weak measuring light (PAR = 0.1 µmol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup>). This way all reaction centres are open (i.e. pigments are not photosynthesising), meaning that the chance of photons being absorbed and utilised for photochemistry is maximum and there is minimal chance of them being dissipated either as fluorescence or heat (Suggett et al., 2010). Measurements of minimal fluorescence can be affected by any remaining electron in  $Q_A$ ; to prevent this, it is sufficient to apply a weak far-red light with which many instruments are equipped (Murchie and Lawson, 2013). Use of far-red light allows a more accurate measurement of minimal fluorescence both in dark-adapted ( $F_0$ ) and light-adapted ( $F_0$ ) conditions. To measure maximum fluorescence ( $F_m$ ), a saturating pulse of actinic light (PAR > 4000 µmol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup>) is applied to a dark-

adapted leaf. With the leaf exposed to a high intensity pulse, all reaction centres are closed (e.g. Murchie and Lawson, 2013) and  $Q_A$  is maximally reduced (e.g. Baker, 2008). The difference between maximum and minimal fluorescence is the variable fluorescence ( $F_v$ ). Butler (1978) demonstrated that the ratio  $F_v/F_m$  is a robust indicator of the maximum quantum efficiency of PSII. This parameter has been found to be consistently close to 0.83 for unstressed leaves of most plant species, with lower values associated with stresses that damage PSII or otherwise reduce fluorescence (quenching) (e.g. Björkman and Demmig, 1987; Baker, 2008; Murchie and Lawson, 2013).

Terms	Description
Actinic light	A type of light that can drive photosynthesis, typical PAR = 500-1000 $\mu$ mol <sub>quanta</sub> m <sup>-2</sup> s <sup>-1</sup> (e.g. Baker, 2008)
Dark-adapted state	Used to describe the state of a leaf that has been kept in the absence of significant PAR, so that it does not photosynthesise, for enough time so that no quenching is present and all PSII centres are open – typically achieved after 20-30 minutes (e.g. Baker, 2008)
Heat loss/dissipation	One of three ways in which a PSII centre can use an absorbed photon (e.g. Murchie and Lawson, 2013)
Light-adapted state	Used to describe the state of a leaf that has been exposed to a given PAR that drives photosynthesis – typically achieved after 20-30 minutes (e.g. Murchie and Lawson, 2013)
Measuring light	Light used for taking fluorescence measurements without driving photosynthesis, typical PAR = $0.1  \mu mol_{quanta} m^{-2} s^{-1}$ (e.g. Baker, 2008)
Non-photochemical quenching	Reduction in fluorescence emission mainly due to an increase in heat loss by PSII (e.g. Baker, 2008)
PAR	Photosynthetically Active Radiation, the component of total spectral irradiance that is photosynthetically active (e.g. Suggett et al., 2010)
Photochemical quenching	Reduction in fluorescence emission due to reaction centres being 'open' after electrons are transferred to quinone acceptor $Q_A$ (e.g. Baker, 2008)
Photoinhibition	Reduction of photosynthetic capacity of PSII (Murchie and Lawson, 2013)
PSII	Photosystem II, a protein complex in which energy is extracted from photons to drive photochemical processes within a leaf (e.g. Kirk, 1994)
PPFD	Photosynthetic Photon Flux Density, equivalent of PAR (Suggett et al., 2010)
Q <sub>A</sub> , Q <sub>B</sub> or quinone acceptors	Protein complexes that have primary role in accepting electrons of PSII (i.e. to transfer energy from PSII to relevant cycles driving plant activities, e.g. Murchie and Lawson, 2013)



Quantum yield (or quantum efficiency)	Ratio of number of molecules used in photochemical processes and number of molecules absorbed by PSII (e.g. Baker, 2008)				
Quenching	Reduction in fluorescence emission associated with changes in the photosynthetic apparatus due to processes including heat loss, photodamage, and closure of reaction centres (e.g. Baker and Rosenqvist, 2004)				
Saturating pulse	Pulse of actinic light that is capable of saturating PSII, typical PAR > 4000 µmol <sub>quanta</sub> m <sup>-2</sup> s <sup>-1</sup> (Murchie and Lawson, 2013)				

Table 1 List of relevant terms, definitions and descriptions.

When a dark-adapted leaf is exposed to actinic light after a saturating pulse, it displays a decrease in fluorescence from  $F_m$  (Figure 4); that is, the fluorescence signal is guenched. After being exposed to a constant intensity of actinic light for 20-30 minutes, a leaf achieves a steady-state and its value of chlorophyll fluorescence in the light can be measured (F). As for a dark-adapted leaf, the value of maximum fluorescence in the light-adapted state  $(F_m)$  can be measured by applying a saturating pulse. The value of  $F_{m'}$  is considerably lower than that of  $F_{m}$  due to non-photochemical quenching processes occurring during photosynthesis (Baker and Rosenqvist, 2004). The photochemical quenching of fluorescence ( $F_a$ ) is the difference between  $F_m$  and F'. The ratio  $F_a'/F_m'$  thus obtained is termed operating efficiency of PSII photochemistry (e.g. Murchie and Lawson, 2013). After a leaf is light-adapted, by switching off the actinic light the minimal fluorescence of the light-adapted leaf  $(F_0)$  is recorded. From these parameters other ratios can be calculated:  $F_0'/F_{\nu'}$ , which gives indication of the level of photochemical quenching of PSII (Murchie and Lawson, 2013); and  $F_v/F_{m'}$ , which provides the maximum efficiency of PSII at a given PPFD (Baker, 2008). Single measurements of fluorescence are affected by both the physicochemical properties of PSII and the optical properties of the leaf, while ratios of fluorescence measurements provide more accurate information, as they cancel out the influence of changes in leaf properties (Baker, 2008). All relevant terms and their definitions are listed in Table 1, and all relevant parameters are defined in Table 2.

Parameter	Definition	Physiological relevance
F, F'	Steady state fluorescence emitted by leaf	None relevant
$F_0$ , $F_0$ '	Minimal fluorescence	Level of fluorescence when all PSII centres are open
$F_m, F_m'$	Maximum fluorescence	Level of fluorescence when all PSII centres are closed
$F_{\nu}$ , $F_{\nu}$	Variable fluorescence calculated as $F_m - F_0$	Linked to the ability of chlorophyll to perform photosynthesis
$F_{q}'$	Difference in fluorescence between $F_{m'}$ and $F'$	Photochemical quenching of fluorescence
$F_{\nu}/F_{m}$	Maximum quantum efficiency of PSII photochemistry	Maximum efficiency of PSII photochemistry

$F_{v}'/F_{m}'$	Maximum efficiency of PSII photochemistry	Maximum efficiency of PSII photochemistry at a given photosynthetically active radiation (PAR)		
$F_{q'}/F_{m'}$	PSII operating efficiency	Estimate of PSII operating efficiency at a given PAR (i.e. proportion of light used in PSII photochemistry)		
$F_{q'}/F_{v'}$ or $qP$	Photochemical quenching	Relates the PSII operating efficiency to the PSII maximum efficiency		
NPQ	Non-photochemical quenching calculated as (F <sub>m</sub> /F <sub>m</sub> ')-1	Provide information on the heat loss from PSII		

Table 2 List of parameters (adapted from Baker, 2008; Murchie and Lawson, 2013). Note that the parameters denoted with a prime (') refer to leaves exposed to actinic light.

#### 3.2.3 Established protocols

A number of protocols or tests to assess plant stress using chlorophyll fluorescence exist. The choice between these depends on the type of stress to which a plant is exposed and the focus of the study (Baker and Rosenqvist, 2004). The most relevant and established tests for ecohydraulics applications are:

- 1.  $F_v/F_m$  test: this test is performed on dark-adapted leaves and requires measurements of  $F_0$  and  $F_m$ . It provides an estimate of PSII maximum quantum efficiency, a parameter that is commonly utilized for assessing plant stress (Baker, 2008; Murchie and Lawson, 2013). In recent years this test has been used for assessing the acclimation of terrestrial plants to environmental conditions (e.g. Janka et al., 2015; Zha et al., 2017) and the effects of light stress on photosynthesis of aquatic plants (Rae et al., 2001; Hanelt et al., 2006; Hussner et al., 2010). A decline in  $F_v/F_m$  and  $F_0$  are accepted to be indicators of photoinhibitory damage in response to high and low temperatures, light stress, and water stress (e.g. Maxwell and Johnson, 2000; Baker and Rosenqvist, 2004). This test can also be used to detect stress caused by  $CO_2$  deficit (Siffel and Braunova, 1999) or extremely low levels of nitrogen (Baker and Rosenqvist, 2004).
- 2. Quenching analysis: these tests aim to characterise either photochemical or non-photochemical quenching (or both). Measurements are taken from light-adapted leaves and include  $F_{m'}$ ,  $F_{0'}$ , and F'. Parameters estimated from quenching analysis such as  $F_{v'}/F_{m'}$  have been used to assess the health status of seagrass (e.g. Durako et al., 2002; Figueroa et al., 2014), freshwater plants (e.g. Hussner et al. 2010), and terrestrial plants (e.g. Janka et al., 2015). These tests require steady-state photosynthesis (i.e. steady light conditions) and can be used to detect drought stress, light stress, heat stress and severe nitrogen stress (Baker and Rosenqvist, 2004). Quenching analysis can be coupled with an  $F_v/F_m$  test run after it to acquire more information about the status of PSII in a leaf.
- 3. Rapid Light Curve (RLC) test: this test was introduced for the first time by White and Critchley (1999) and is used to assess the photosynthetic performance of a plant as a function of light irradiance. While irradiance is increased gradually measurements are taken in an unsteady state; that is, leaves are not fully adapted to the light conditions (Ralph and Gademann, 2005). This test gives a reliable assessment of photosynthetic activity in light-fluctuating conditions that are common underwater. Therefore, RLC has been widely used to study seagrass in its natural environment (e.g. Ralph and Gademann, 2005; Belshe et al., 2007; Durako, 2012). This test can



be used to describe the photosynthetic capacity of a leaf under a range of light conditions, its capacity to tolerate light changes and light-adaptation state (Ralph and Gademann, 2005).

Among these tests, the  $F_v/F_m$  test appears to be the most appropriate for monitoring the health status of vegetation in flume facilities for several reasons. First, the measurements are independent of the light conditions to which leaves are exposed and provide more general information on plant health. Second, even though it requires leaves being dark-adapted prior to measurements, it is the simplest test to conduct. Third, it yields a single parameter that can be easily understood by hydraulic researchers without a biological background: for most species, unstressed leaves have values of  $F_v/F_m$  equal or close to 0.83, and lower values are measured from leaves that have been exposed to stress (Björkman and Demmig, 1987; Baker, 2008; Murchie and Lawson, 2013).

#### 3.3 Examples of Instruments

Thanks to technological developments and increasing understanding of plant physiology, the use of chlorophyll fluorometers is becoming more and more common and more sophisticated instruments have been designed for general use. Chlorophyll fluorometers are manufactured by several specialised companies such as ADC BioScientific Ltd, Aquation Pty Ltd, Hansatech Instruments Ltd, Photon Systems Instruments, and Walz. Different types of instruments are available within a price range from 5000 through to 30000. The most expensive are usually equipped with multiple sensors and/or a PAR sensor and characterised by multiple functionalities. For ecohydraulics applications, an instrument is needed that can measure parameters such as  $F_v/F_m$ ,  $F_v'/F_{m'}$ , NPQ and perform most established tests using automated procedures. Some instruments can be controlled via PC interface but most of them are interfaced via a device or console. Other considerations include: presence (or absence) of a leaf clip and PAR sensor; if the leaf clip is designed for dark-adaptation; if the fluorometer is equipped with a far-red light; if the irradiance of actinic light can be adjusted; and waterproofness. It is worth noting that most fluorometers do not include leaf clips, which must be acquired separately.

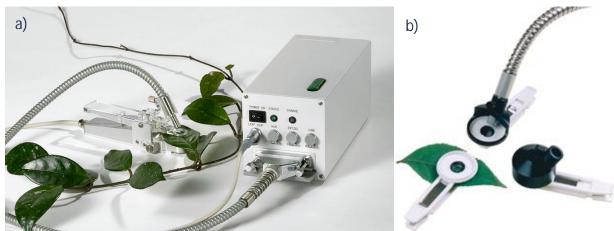


Figure 5 (a) Example of chlorophyll flouorometer consisting of main device that can be connected to an interface (e.g. PC or tablet), a fibre otpic cable and leaf clip (adapted from www.walz.com); (b)examples of leaf clips that can be used to hold leaves and dark-adapt them (adapted from www.hansatech-instruments.com).



# 4 APPLICATION OF CHLOROPHYLL FLUORESCENCE ANALYSIS IN FLUME FACILITIES

## 4.1 DESCRIPTION OF THE $F_V/F_M$ TEST

As stated in Section 3.2.3, the  $F_v/F_m$  test has been extensively used to detect plant stress associated with damage to the photosynthetic apparatus and caused by factors such as drought, light, and heat (Baker and Rosenqvist, 2004). When conducting experiments, however, one should bear in mind that environmental factors that impact upon PSII, directly or indirectly, will also impact measurements of  $F_v/F_m$  (Wozniak et al. 2002). The test is conducted on dark-adapted leaves and provides an estimate of PSII maximum quantum efficiency ( $F_v/F_m$ ) from measurements of the minimal fluorescence ( $F_o$ ) and the maximum fluorescence ( $F_m$ ). Measuring in the dark provides an advantage in that there is no need to be concerned about the sample being partially shielded by the fibre optic cable or leaf clip as is the case during light-adapted measurements. Therefore, the distance between the fibre optic cable and the leaf and the inclination of the cable with respect to the leaf do not play a significant role in this test.

Prior to conducting an  $F_v/F_m$  test, a few crucial arrangements should be made:

- 1) <u>Identification of leaves to be used for measurements</u>. As a rule of thumb in botany, the youngest mature leaves should be used for diagnosis (Reuter and Robinson, 1997).
- 2) <u>Identification of part(s) of a leaf to be used</u> (note that if imaging PAM is utilized this does not apply). This issue is important because of the heterogeneity of the chlorophyll fluorescence signal across a leaf (e.g. Murchie and Lawson, 2013). Kalaji et al. (2014) highlight that in dorsiventral leaves (i.e. leaves with different ventral and dorsal surfaces) the characteristics of the top and bottom surfaces differ as light is mainly absorbed by the top part.
- 3) Selection of method and time of dark-adaptation. Leaves can be dark-adapted by using a leaf clip for shielding them from ambient light, keeping them under laboratory (dark) conditions, or performing measurements before dawn (e.g. Kalaji et al., 2014). There is no clear guideline regarding dark-adaptation time; 30 minutes is indicated as a generally acceptable time, but predawn measurements are advisable in the field and for plants under severe stress (Murchie and Lawson, 2013; Kalaji et al., 2014).
- 4) <u>Setup of the instrument.</u> The instrument should be set up so that it takes into account any background fluorescence from the environment (i.e. zeroing) and the gain and intensity of both light sources (measuring and actinic) are adjusted depending on the plant species. The exact procedure for this step depends on the chlorophyll fluorometer in use.

After a leaf is dark-adapted appropriately and the previous arrangements have been made, the test can be conducted. It is crucial that the leaf is kept in darkened conditions throughout the entire test. First, if the instrument is equipped with a far-red light, the leaf should be exposed to a short and weak pulse of far-red light to allow a more accurate measurement of  $F_0$ . Second, minimal fluorescence is measured using a measuring light with PAR of about 0.1  $\mu$ mol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup>. Third, a strong saturating pulse (PAR > 4000  $\mu$ mol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup>) of short duration (i.e. less than 1 second) is applied and maximum fluorescence measured multiple times. Finally, the user should ascertain that the maximum fluorescence reached during the saturating pulse is not quenched by checking that the



multiple values of  $F_m$  are approximately constant (i.e. not decreasing with time) (Murchie and Lawson, 2013).

This set of instructions, that can be extracted from relevant literature, may be sufficient for endusers with a biological background and some experience using chlorophyll fluorometers. However, it does not provide enough practical information to allowing hydraulic researchers to directly apply this technique and test live vegetation in flume facilities. Some important issues that can limit the use of chlorophyll fluorescence remain and common questions might be:

- How do I identify the youngest mature leaves in a plant?
- How do I select and use leaves in plants with compound leaves (i.e. in which the blade consists of multiple leaflets or blades) such as ferns?
- How do I measure chlorophyll fluorescence on leaves that are too small to cover the fibre optic cable cross-section completely?
- In which part of a leaf should chlorophyll fluorescence be measured?
- For how long should a leaf be dark-adapted?

There is, therefore, a need to establish pragmatic guidelines for hydraulic researchers to use. We conducted some methodological experiments, which are described in the next section, that are intended to provide further practical guidance around these issues. The focus of these experiments was on: (i) working with leaves with different morphological traits; (ii) identifying a good dark-adaptation time; (iii) assessing the effect of the location of the leaves along a plant on the measurements; and (iv) identifying the most reliable location for measurements within a leaf.

#### 4.2 HYDRALAB+ METHODOLOGICAL EXPERIMENTS

The experiments described in this section were conducted in the River Science laboratory of Loughborough University and using a Classic Fluorometer (Aquation Pty Ltd, Umina Beach, Australia).

#### 4.2.1 Role of leaf morphology

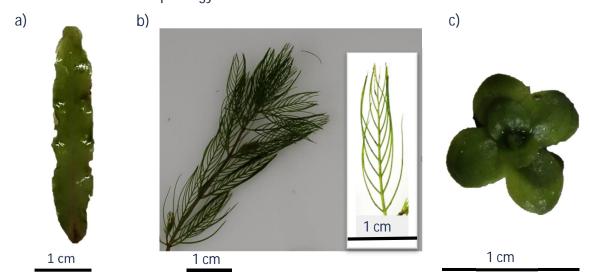
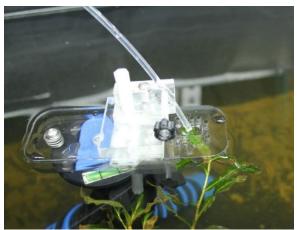


Figure 6 Different morphological traits shown by leaves of (a) P. crispus, (b) M. verticillatum, and (c) C. stagnalis. Inset of (b) displays an individual leaf of M. verticillatum with 16 leaflets.



Mesocosm experiments to evaluate the effectiveness and feasibility of the  $F_{\nu}/F_{m}$  test were conducted in summer 2017. Experiments were carried out with three freshwater macrophyte species: Callitriche stagnalis, Myriophyllum verticillatum, and Potamogeton crispus. These species were selected based on availability, widespread distribution across Europe and diversity of leaf morphology (Figure 6). P. crispus have linear or oblong leaves a few cm long (Figure 6a). M. verticillatum leaves are compounded with multiple needle-like leaflets that make them look like feathers (Figure 6b). Top leaves of C. stagnalis are compounded with spoon-shaped leaflets grouped into rosettes (Figure 6c).



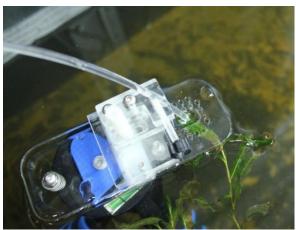


Figure 7 Fibre optic cable, leaf clip, and leaf of P. crispus during measurements.

The health status of plants was assessed under a range of environmental conditions (Table 3) by monitoring their chlorophyll fluorescence daily for 5 days. Environmental conditions were set based on the outcomes of a survey which we conducted among ecohydraulics researchers in May and June 2017. The survey was completed by 26 researchers and included questions about: the time during which vegetation is kept in flume facilities, the light conditions to which vegetation is exposed, the water temperature, and the type of water used. Based on the feedback received from our colleagues, we designed six treatments listed in Table 3 (with two treatment levels for each factor: water type, water temperature and light) in such a way that they replicated the typical conditions to which vegetation is exposed in flume facilities. Each treatment mesocosm consisted of an 80 I plastic container filled with water up to a depth of 28 cm. Water was aerated using air pumps and airstone bars to guarantee an optimal aeration distribution. To start each treatment, eight plants were randomly selected from the storage tanks and plastic labels were applied on them so that they could be identified during the monitoring phase. Plants were then inserted in the dedicated mesocosm and homogeneously distributed throughout the container to prevent them from shading. Plants were exposed to treatments for 5 consecutive days and their health status was monitored daily starting from 24 hours after a treatment started. At the end of a treatment, plants were removed from the mesocosm and mechanical tests were conducted with specimens prepared from their stems to investigate their biomechanical properties.

The  $F_{\nu}/F_m$  test was conducted before dawn on the youngest fully mature leaf of each plant, the same leaf (or leaflets) was used throughout the experiments. During measurements plants were exposed to PAR < 0.2  $\mu$ mol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup>, which would not trigger photosynthetic activity in the leaves, as recommended by Baker and Rosenqvist (2004). The way in which each leaf was identified and chlorophyll fluorescence measured depended on the morphological traits of each species. For *P. crispus* each leaf was chosen so that it showed morphological features (e.g. shape and colour) typical



of fully grown leaves in the same plant, but it was located on the top part of the plant (Figure 7). Chlorophyll fluorescence was then measured in the centre of the leaf. For *M. verticillatum* a single leaflet was too small to cover a considerable portion of the sampling area (i.e. of the size of the fibre optic cable cross-sectional area), therefore one or more leaves located close to the top of a plant were used (see inset of Figure 6b for visualization of a leaf and leaflets). To do so, leaflets were clumped up so that they would cover the sensor sampling area. In this case it was not possible to select a specific part of the leaf for measurements. Similar to *M. verticillatum*, leaflets of *C. stagnalis* were not sufficiently big to be used individually for measurements. For this reason, measurements were taken on two or three adjacent leaflets so that the sampling area was covered by them. Due to the reduced size of leaflets, whole leaflets were included in the sampling area.

Treatments	Type of water	Water temperature (°C)	Light irradiance (µmol <sub>quanta</sub> m <sup>-2</sup> s <sup>-1</sup> )	Conductivity (µs/cm)	рН
Pond Water	Pond water	16-22	120-180	540-608	8.5-8.71
Tap Water	Tap water	16-22	120-180	632-674	8.38-8.61
Low Temperature	Pond water	12-13	120-180	530-637	8.55-8.74
High Temperature	Pond water	25-32	120-180	603-740	8.6-8.68
Low Irradiance	Pond water	18-23	1-2.5	611-681	8.58-8.71
High Irradiance	Pond water	18-29	325-375	569-643	8.55-8.78

Table 3 Description of the treatments to which freshwater macrophytes were exposed. Temperature, irradiance, conductivity and pH were measured daily, range of values is reported for each parameter.

Notwithstanding the differences in leaf morphological traits, the  $F_v/F_m$  test was successfully conducted on all species. The values of  $F_v/F_m$  for unstressed plants – measured within 24 hours of delivery to the River Science laboratory on plants not exposed to obvious abiotic stresses – were close to values indicated by the relevant literature. *P. crispus* and *C. stagnalis* showed mean values of  $F_v/F_m$  between 0.78 and 0.83, while *M. verticillatum* showed a mean value of  $F_v/F_m$  close to 0.73. We believe that values of  $F_v/F_m$  for *M. verticillatum* were considerably lower than 0.83 because experiments with this species were conducted at the end of the growing season, when plants experience non-optimal conditions. Experiments with *P. crispus* and *C. stagnalis* were performed in mid-August and early September respectively, whereas samples of *M. verticillatum* were tested in early October.

The results of the experiments indicate that environmental conditions typical of hydraulic laboratories can induce significant stress in plants, with significant reductions in health status. As an example, we report here on the results from experiments with *P. crispus*. Samples of *P. crispus* in the 'Pond Water' treatment, which was designed to minimize plant stress, experienced a significant reduction in  $F_v/F_m$  (t-test, mean slope = -0.01 d<sup>-1</sup>, df=7, p << 0.01). Similarly, the other treatments negatively affected plant health. In some cases leaves were so deteriorated that  $F_0$  was below instrument accuracy. In such cases, the measurement was invalid and we assumed  $F_v/F_m = 0$ , because this corresponds to the poorest health status. We then calculated the mean daily value  $(F_v/F_m)$  of  $F_v/F_m$  and compared the trend in time for each treatment (Figure 8a). It is evident in Figure 8a that 'Tap Water' and 'Low Irradiance' treatments are associated with reduction in health considerably higher (slope = -0.12 to -0.13 d<sup>-1</sup>) than that for the remaining treatments (slope = -0.01

to -0.02 d<sup>-1</sup>). The linear regression of  $F_v/F_m$  was then computed for each plant in the remaining treatments using the Least Square Difference method (Figure 8b). Comparing the slopes of the linear regressions across treatments using Tukey's Honest Differences test corrected using Bonferroni adjustment, we found that 'Low Temperature' is associated with a greater reduction in  $F_v/F_m$  than that for the three remaining treatments (p = 0.06-0.13), which are statistically indistinguishable from one another (Figure 8b). Across the three species used for our experiments, exposure to low irradiance and unconditioned tap water appeared to be the most stressing treatments. Furthermore, samples of *P. crispus* exposed to 'Low Irradiance' and 'Tap Water' were visibly stressed.

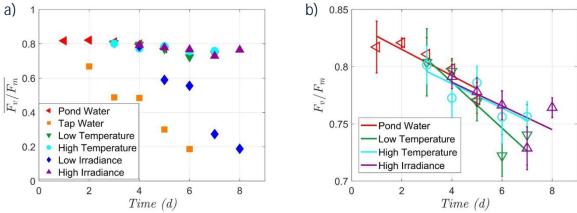


Figure 8 (a) Mean (across plants) daily values of  $F_v/F_m$  for P. crispus for each treatment. (b) Linear regression of  $F_v/F_m$  for P. crispus calculated using the mean intercept and slope for each treatment (markers represent mean values, bars are twice the standard deviation). Note that in (b) treatments characterised by invalid measurements are not shown.

At the end of a treatment specimens for mechanical tests were prepared from plants. Mechanical tests at bending and tension were performed on specimens prepared from the top and the bottom of the stems. From each plant four specimens were prepared, two from the top part and two from the bottom part, in each case one specimen was used for tensile tests and one for flexural tests. From the mechanical tests we obtained estimates of Young's modulus at tension and bending ( $E_t$  and  $E_h$ ) and flexural rigidity ( $E_h$ ). Since biomechanical properties can vary along a plant (e.g. Miler et al. 2014), specimens prepared from the top and the bottom of plants were analysed separately. By using analysis of variance, we compared values of biomechanical properties from plants within each treatment with those of specimens cut from a 'Control' group of unstressed plants (i.e. tested on the 1<sup>st</sup> day of experiments, the day in which treatments started). We performed six pairwise comparisons for top specimens and six pairwise comparisons for bottom specimens and corrected the significance level using Bonferroni adjustment (significance level set to 0.05) to assess if some treatment affected plant biomechanics. We found that some treatments were associated with a significant change in biomechanical properties, even though changes were species dependent (Table 4). The magnitude of changes varied from a 66% decrease to a 48% increase, and almost all changes (but one) indicated a decrease in stiffness or rigidity. Importantly, treatments reported to cause the most stress to plants were also associated with significant changes in plant biomechanical properties. However, no evident relationship was found between the values of  $F_v/F_m$  and the changes in plant biomechanical properties.

The  $F_v/F_m$  test was successfully employed to monitor the health status of three species of freshwater macrophytes exposed to a range of environmental conditions. The results of these methodological experiments indicate that measuring chlorophyll fluorescence from a single young mature leaf as



indicator of health status for a plant can cause a bias in the assessment of plant health. Indeed, this sampling method does not consider the status of the remaining leaves and, therefore, cannot be considered as representative of the entire organism. Furthermore, reduction in values of  $F_v/F_m$  across treatments reveal that typical environmental conditions to which plants are exposed in flume facilities stress plants considerably and can have a significant effect on plant biomechanical properties even within a short temporal scale.

Species	Plant part	Young's modulus at tension - $E_t$		Young's modulus at bending - $E_b$		Flexural rigidity - E <sub>b</sub> I	
		Treatment	Variation	Treatment	Variation	Treatment	Variation
D crienus	Тор	Low Temp.	-35%			Low Irrad.	-66%
P. crispus	Bottom	Tap Water	+48%				
	Тор			High Temp. Low Irrad. High Irrad.	-43% -43% -45%	High Temp. Low Irrad. High Irrad.	-41% -58% -53%
C. stagnalis	Bottom			Low Temp. High Temp. Low Irrad. High Irrad.	-34% -41% -35% -57%		
M. verticillatum	Тор	Low Irrad.	-29%	Tap Water Low Irrad.	-49% -35%	Tap Water	-58%
	Bottom			Low Temp.	-42%		

Table 4 Summary of cases in which environmental conditions induced significant changes in plant biomechanics. Cases are defined considering species, plant part, treatment to which the plant was exposed and biomechanical property. All cases included in the table are characterised by a p-value lower than 0.05.

#### 4.2.2 Role of dark-adaptation time and inter-leaf and intra-leaf variation

Additional methodological experiments were conducted with the following aims: (1) to establish where measurements of  $F_v/F_m$  should be taken along a leaf; (2) to establish on which leaves along a plant measurements of  $F_v/F_m$  should be taken; (3) to estimate what is the minimum dark-adaptation time required to get accurate estimates of  $F_v/F_m$ . Mesocosm experiments with P. crispus were conducted in May 2018, plants were divided into four dark-adaptation groups: 1 night, 1 hour, 15 minutes, 2 minutes. These dark-adaptation times were selected to include values recommended in the literature and values that appear to be more feasible in hydraulic experiments. To limit any adverse effects of abiotic factors on plant health status, before measurements plants were stored for up to 48 hours in aerated containers with water temperature equal to 16°C and exposed to an irradiance of about 150  $\mu$ mol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup> at the water surface. Ten random plants from each group were selected, chlorophyll fluorescence was measured on five young mature leaves for each plant and at three locations on each leaf: close to the petiole (bottom), in the centre, close to the apical end (top). Leaves were chosen at various positions along a plant defined by the number of internodes from the top of the plant (see Figure 9 for definition of internodes).



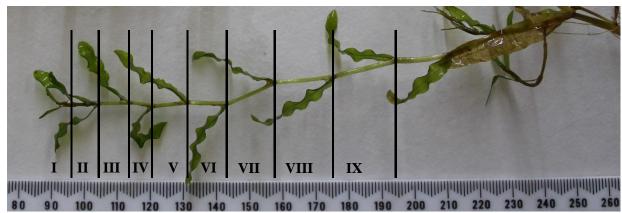


Figure 9 Stem of P. crispsus subdivided into internodes. An internode is defined as the space along the stem between nodes, which are the region of a stem from which one or more leaves or branches emerge (e.g. Graham et al., 2006). In the case of P. crispus nodes can be easily identified by the change in stem morphology.

Initial data analysis showed that measurements of  $F_{\nu}/F_m$  taken from the bottom of leaves were characterised by a variance considerably higher than those from other parts of leaves, independent of other factors. For this reason, data from the bottom of leaves were not considered in further analysis. Linear mixed effect models were then used to evaluate the effect of each factor on  $F_{\nu}/F_m$  and indicated that all three factors (i.e. dark-adaptation time, location of leaves within a plant, and location of measurement along a leaf) had a significant effect on  $F_{\nu}/F_m$  measurements.

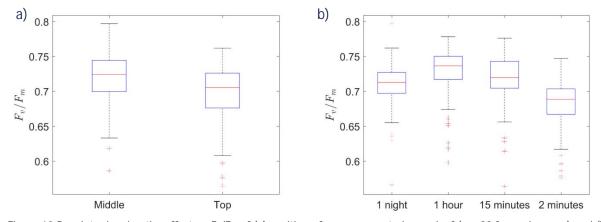


Figure 10 Boxplots showing the effect on  $F_v/F_m$  of (a) position of measurement along a leaf (n = 82 for each group) and (b) dark-adaptation time (n = 41 for each group). The boxplots have lines at the lower and upper quartile (blue box) and median (red line), whiskers extend to +/-2.7 times the standard deviation, and red crosses are outliers.

By considering only those leaves located within the top three internodes (Figure 9) the effect of the location of leaves within a plant became insignificant and could be removed from the model. The dark-adaptation time and location of measurement within a leaf remained significant, while their interaction could be neglected. To analyse the specific effects of these two factors, multiple comparison analysis was performed using Tukey's test with Bonferroni adjustment. This analysis showed that a 15-minute dark-adaptation time is the most appropriate choice, because results did not differ significantly from those obtained with a 1-night or 1-hour dark-adaptation (Figure 10b). On the other hand, 2-minute dark-adaptation was significantly different from the other groups ( $F_{3,394} = 29.1$ , p << 0.01). Regarding the location of measurement within a leaf, values of  $F_{\nu}/F_{m}$  from the middle of a leaf were significantly different from those from the top ( $F_{1,396} = 39.9$ , p << 0.01, Figure

10a). Considering this result and the practicalities of clipping a leaf, taking measurements in the middle part of a leaf is the most straightforward and preferable choice.

### 4.3 A PROTOCOL FOR USING CHLOROPHYLL FLUORESCENCE ANALYSIS IN FLUME STUDIES

Based on relevant literature and the results from our methodological experiments, this section describes a methodological protocol for the application of chlorophyll fluorescence analysis to assess the health status of live vegetation in flume facilities in the form of step-by-step guidelines. The focus is on the  $F_v/F_m$  test that was comprehensively described in section 4.1. Some parts of the protocol were established based on methodological experiments conducted with  $P.\ crispus$ ; while we expect them to be applicable to a wide range of plants, we recommend practitioners that use different species to perform similar methodological experiments to establish suitable practical guidelines valid for those species. The protocol can be sub-divided into three main parts: design, preparation, measurements.

#### Design:

- 1. Once a plant species is selected search the literature for studies of plant physiology referring to the same species. This can be useful for identifying the optimal conditions for a species, benchmark values for chlorophyll fluorescence parameters, and a consistent and established procedure to follow during experiments. If plants being used in laboratory experiments are collected from the field, benchmark values for the most important chlorophyll fluorescence parameters can be estimated by measuring chlorophyll fluorescence on plants in situ before collection.
- 2. For designing  $F_v/F_m$  tests, consider morphological traits of both the plant's main structure and its leaves. As a rule of thumb, the youngest mature leaves should be used. More pragmatically, leaves showing morphological characteristics typical of the species and located on the top three internodes should be used. Note that this recommendation is not applicable to all plant species, for example in studies of seagrass using the  $2^{nd}$  innermost (and therefore  $2^{nd}$  youngest) blade is a relatively well-established procedure (e.g. Durako et al., 2012).
- 3. Considering the typical size of leaves, select how many leaves are required to take a measurement and/or what part of a leaf should be used. The central part of a leaf (where applicable) should be used as it provides more consistent data. For compound leaves, if an individual leaflet is not sufficiently large to cover the whole sampling area, leaflets can be clumped together. As long as ratios of chlorophyll fluorescence measurements (e.g.  $F_v/F_m$ ), are used in the analysis the number of leaflets within the sampling area does not affect the results.
- 4. Think whether leaves should be detached from plants for measurements or not. The physiological characteristics of leaves change within hours of detachment (e.g. Weng, 2011), but detached leaves can be kept in water (for aquatic plants) or wet filter paper (for terrestrial plants) during their dark-adaptation. Considerations regarding these issues are dependent on the spatial and temporal scale of the experiments and the size of plants used.
- 5. The requirements of intended statistical testing (for example, the need for independent measurements) should also be considered at this stage.



6. For the particular facility's light conditions, identify a way to dark-adapt samples for 15 minutes before chlorophyll fluorescence measurements are taken. Also, consider that measurements of  $F_0$  and  $F_m$  must be taken in the dark.

#### Preparation:

- 1. Following the instructions in the user manual, set up the chlorophyll fluorometer and 'zero' it with the light conditions in which the measurement will take place (and without a leaf on the leaf clip).
- 2. Most instruments are supplied with predefined settings that are intended to provide good results irrespective of the species. However, we recommend that users find the best settings to perform measurements on the selected species by testing the instrument with some leaves from that species. As a rule of thumb, Kalaji et al. (2014) indicate that the value of  $F_0$  should be close to 10% of the range of the instrument. However, it is crucial that the values of chlorophyll fluorescence measured lie within the range of the instrument. To optimise the instrument settings the user can vary sensor gain, the intensity of saturating and measuring lights, and the distance between the sample and the end of fibre optic cable.

#### Measurements:

- 1. Once the instrument is set up, insert the sample in the leaf clip so that the sampling area is covered by it.
- 2. Expose the sample to a short and weak pulse of far-red light and measure  $F_0$  using a measuring light with PAR of about 0.1  $\mu$ mol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup>. Note that an automated protocol for this measurement should be available on the instrument.
- 3. Apply a strong saturating pulse (PAR > 4000  $\mu$ mol<sub>quanta</sub>m<sup>-2</sup>s<sup>-1</sup>) for less than one second and measure maximum fluorescence ( $F_m$ ). Once again, an automated protocol for this measurement should be available on the instrument.
- 4. After measurements have been taken, check that the value of  $F_m$  reached during the saturating pulse is not quenched (Murchie and Lawson, 2013). To do this, check the chlorophyll fluorescence trace, multiple values of  $F_m$  should be present and they should be approximately constant (i.e. not decreasing with time).
- 5. Finally, if the instrument does not apply this filter automatically, remove all readings of  $F_0$  (and the corresponding  $F_m$ ) that are below the acceptable minimum of the instrument range. To estimate the acceptable minimum, consult the user manual or the instrument manufacturer.

#### 4.4 OTHER CONSIDERATIONS

Practitioners may still be challenged by a number of practical issues that have not been dealt with in the previous sections:

- Leaves from different environments are adapted to different conditions, caution is therefore required when comparing their chlorophyll fluorescence traces (Kalaji et al., 2014).
- Leaves of considerable size (e.g. from trees) are likely to display a strong heterogeneity of chlorophyll fluorescence across their surface. To obtain values of chlorophyll fluorescence that are representative of entire leaves, multiple measurements on each leaf may be required (this does not apply if imaging PAM fluorometer is used).

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- Leaves growing on different sides or parts of a plant are likely be exposed to different light conditions (particularly in large plants); this should be taken into account when selecting leaves in such a way that they are representative of the entire organism. This also means that if a considerable number of severely deteriorated leaves are present they should be considered in the analysis (e.g. by setting their  $F_V/F_m$  null).
- For identifying the youngest mature leaves, it is fundamental to understand plant growing mechanism. For example, in some plants new leaves grow on the apical parts of stems, in others, such as grass or seagrass, new leaves grow in the inner part of the sheath. Other organism such as seaweeds, or macroalgae, can have blades that grow from either the top or bottom part.
- Seasonality plays a crucial role in the vital cycle of many plants, particularly those adapted to temperate or sub-arctic climates, dramatically affecting their photosynthetic activities.
- When clipping a leaf, the sampling area of the sensor should be entirely covered. While this
  is not an absolute requirement when ratios of chlorophyll fluorescence measurements are
  analysed, samples not covering the entire sampling area will provide lower absolute values
  of chlorophyll fluorescence.
- The leaf clip might not shield the sampling area properly if using very ruffled leaves (some stray light could be present), or smooth leaves (leaf clip may shift or slide, Kalaji et al., 2014).
- If it is necessary to repeat a measurement, bear in mind that the tissues exposed to a saturating pulse to measure  $F_m$  will be affected by the previous measurement. It is, therefore, recommended that a different sampling area is selected.
- If measuring chlorophyll fluorescence in the light-adapted state, shielding must be prevented (e.g. Durako et al., 2012), including consideration of the angle and distance between the fibre optic cable and leaf.

## 5 Examples of applications

The validity and effectiveness of the protocol outlined in section 4.3 was tested by employing it to assess the health status of the aquatic macrophyte *P. crispus* in three additional sets of experiments. As in Section 4, these experiments were conducted in the River Science laboratory of Loughborough University and using a Classic Fluorometer (Aquation Pty Ltd, Umina Beach, Australia).

## 5.1 EFFECT OF LABORATORY STORAGE CONDITIONS ON HEALTH STATUS OF *P. CRISPUS*

The results of methodological experiments previously described indicated that, among environmental conditions typical of hydraulic laboratories, exposure to low irradiance and unconditioned tap water were the most stressful conditions for freshwater macrophytes in a 5-day period. Based on these results, mesocosm experiments were conducted in May 2018 to investigate the changes in the health status of *P. crispus* associated with these conditions throughout a longer period of 13 days. At the beginning of the experiments 120 shoots were allocated to each of the treatments described in Table 5. The 'Pond Water' treatment was designed so that it would replicate optimal conditions for *P. crispus*. Every day, eight shoots were removed from each treatment and their health status was assessed by measuring chlorophyll fluorescence on the middle part of a young mature leaf located within the top three internodes. After measurements, shoots were discarded.

Treatments	Type of water	Water Temperature (°C)	Light Irradiance (µmol <sub>quantum</sub> m <sup>-2</sup> s <sup>-1</sup> )	Conductivity (µs/cm)	рН
Pond Water	Pond water	18-21	150-180	535-641	8.5-8.7
Tap Water	Tap water	18-20	130-170	580-619	8.3-8.7
Low Irradiance	Pond water	18-21	1-2.5	638-678	8.5-8.6

Table 5 Description of treatments used for mesocosm experiments. Temperature, irradiance, conductivity and pH were measured daily, range of values is reported for each parameter.

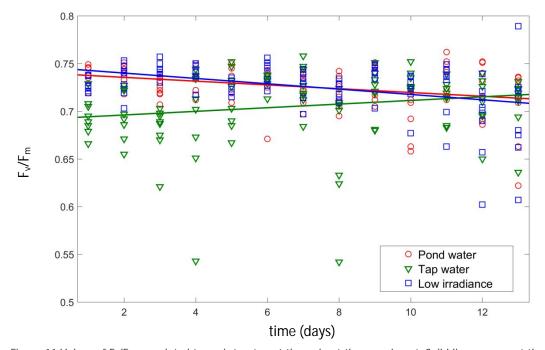


Figure 11 Values of  $F_v/F_m$  associated to each treatment throughout the experiment. Solid lines represent the best linear fit for each treatment.

Data of  $F_v/F_m$  were analysed using analysis of covariance to assess the effect of each treatment in time (Figure 11). Results showed that there are differences in the health status of shoots exposed to the 'Tap Water' treatment and those exposed to the remaining treatments. Health status of shoots in the 'Tap Water' treatment was significantly lower than other treatments at the beginning of the experiments (p << 0.01), indicating that this condition induced an acute (i.e. short term) stress on shoots. In the longer term, however, the trend was the opposite and the slope of  $F_v/F_m$  was significantly higher for the 'Tap Water' treatment than for the remaining treatments. This suggests that once acclimated to the tap water, shoots recovered their health. In contrast, the health status of shoots exposed to the 'Low Irradiance' or 'Pond Water' treatments deteriorated with time.

These results are in contrast with findings from methodological experiments described in section 4.2.1, where tap water and low irradiance alike were found to cause severe stress to plants. The most likely reason for this divergence is to be found in seasonality. Methodological experiments with *P. crispus* described in section 4.2.1 were conducted in mid-August, whereas the experiments described here were conducted in mid-May. *P. crispus* is known to have an early growing season



compared to most freshwater macrophytes, with a growth peak in late spring or early summer (Nichols and Shaw, 1986). When the growing season ends, between late June and August, *P. crispus* undergoes a quick decline (Nichols and Shaw, 1986). Therefore, we believe that plants used in methodological experiments were more sensitive to abiotic stresses because they were at the end of the growing season, whereas plants used in these experiments were more resilient. This result highlights the important role of seasonality in defining plant health under different conditions and indicates the need for researchers to consider plant seasonality when designing experiments for preventing bias associated with this factor.

## 5.2 EFFECT OF HYDRAULIC CONDITIONS ON HEALTH STATUS OF *P. CRISPUS*

Flume experiments were conducted in June 2018 to assess the effect of a range of hydraulic conditions on the health status of *P. crispus*. Prior to flume experiments plants were kept for up to seven days in a storage tank and exposed to environmental conditions as per 'Pond Water' treatment described in Table 5. Plants were tested at five flow scenarios, with mean flow velocity ranging from 0.07 to 0.53 m/s and constant light conditions (Table 6). For each flow scenario, 16 plants were randomly selected from the storage tank and eight of these were fixed on the flume bed by means of cable glands glued to the flume bed (Figure 12). The remaining plants were located in a control tank with the same environmental conditions as the flume but no flowing water. The control tank was set up using water from the flume so that it contained Acoustic Doppler Velocimeter (ADV) seeding, it was exposed to the same light conditions as the flume (see Table 6) and it was equipped with an aeration system. The flume was not equipped with an aeration system because it was assumed the aeration generated by pump, inlet and outlet was sufficient. Plants were exposed to these conditions for approximately seven hours after which time they were removed from the flume or tank and their health status was assessed by measuring  $F_v/F_m$  as described in section 4.3. In this case  $F_v/F_m$  of a plant was measured on the middle part of eight young mature leaves located within the top three internodes. For values of  $F_v/F_m$  to be representative of the entire plant, the eight leaves were selected so that the number of leaves from each stem was approximately the same. After each experiment plants were discarded. Comparing values of  $F_v/F_m$  for plants located in the flume and those located in the control tank allowed an assessment of the effect of mean flow velocity on plant health status.

Flow scenario	Bed slope - s (%)	Flow rate - Q (I/s)	Water depth - d (m)	Mean flow velocity - U (m/s)	Light irradiance (µmol <sub>quanta</sub> m <sup>-2</sup> s <sup>-1</sup> )
Run 1	0.75	22	0.29	0.07-0.12	7
Run 2	0.75	34	0.29	0.15-0.23	7
Run 3	0.8	45	0.29	0.22-0.27	7
Run 4	1	58	0.30	0.27-0.34	7
Run 5	1	76	0.29	0.38-0.53	7

Table 6 Description of (hydraulic and light) conditions for each flow scenario. Note that mean flow velocity is reported as the range of mean flow velocities experienced by all plant across and along the experimental section.

Linear mixed effect models were used to assess the effect of flow scenarios and treatments (i.e. control tank and running water) on values of  $F_v/F_m$ . We found that flow scenario was the most significant factor (p << 0.01), but also treatment (p = 0.08) and the interaction (p < 0.01) between the two factors were significant. Data of  $F_v/F_m$  were further analysed using analysis of variance to



compare the health status of plants located in the control tank (n=8) and those in the flume (n=8) for each flow scenario independently. As expected from results of linear mixed effect models, running water had a different effect on plant health depending on the mean flow velocity (Figure 13a). For the flow scenario 'Run 1' the values of  $F_v/F_m$  for the control were significant higher (p = 0.06), for the scenario 'Run 2' there was no significant effect of treatment (p = 0.52), for the remaining scenarios the values of  $F_v/F_m$  for the control were lower than for the running water ('Run 3', p < 0.01; 'Run 4', p = 0.18; 'Run 5', p = 0.05).

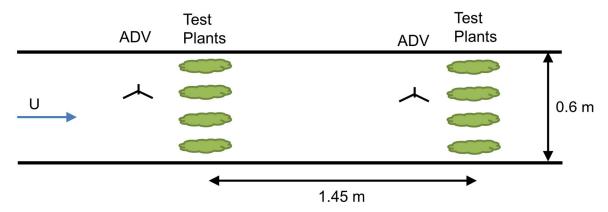


Figure 12 Sketch of the experimental setup (top view). U is the mean flow velocity in front of the upstream row of plants.

However, similar to what reported in section 4.2.1, at the end of flume experiments some of the plants presented stems without leaves or with leaves so deteriorated that no valid measurements of  $F_{\nu}/F_{m}$  could be obtained. To account for this, analysis was performed also setting the value of  $F_{\nu}/F_{m}$ arbitrarily to zero for any invalid measurement, because this corresponds to the poorest health status. To prevent non-equality of variance in the data ensuing, the mean value  $(\overline{F_v/F_m})$  of  $F_v/F_m$ across each plant was used to compare the health status of plants located in the control tank (n=8) and those in the flume (n=8) using analysis of variance. Results of this analysis indicated that values of  $\overline{F_v/F_m}$  were significantly lower for plants exposed to running water for flow scenario 'Run 5' (p = 0.03) and marginally for flow scenario 'Run 4' (p = 0.25), while no significant effects were registered for scenarios with lower mean flow velocities (Figure 13b). It is evident that these results are in contrast with those obtained analysing only valid values of  $F_v/F_m$  (Figure 13a). Rather than being directly related with plant photochemistry, however, these results appear to be caused by weaker leaves being stripped off/dislodged from plants when they are exposed to high flow velocities. Indeed, the number of leaves missing in plants located in the flume was considerably higher than that in plants allocated to the control tank in flow scenarios 'Run 4' (no leaves missing in control tank, 7 leaves missing in flume) and 'Run 5' (12 leaves missing in control tank, 23 leaves missing in flume).



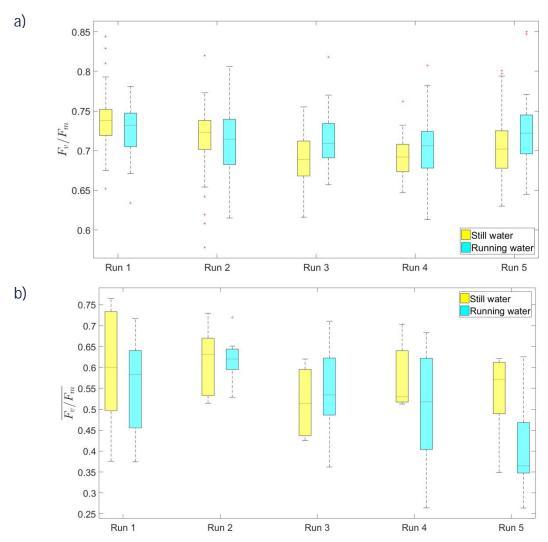


Figure 13 Boxplots showing the effect of flow scenarios on  $F_v/F_m$  (a) and on the mean values of  $F_v/F_m$  across plants (b). For each flow scenario in (a) n = 128 (of which 64 were in the control tank and 64 in the flume), for each flow scenatio in (b) n = 16. The boxplots have lines at the lower and upper quartile (yellow or cyan box) and median (red line), whiskers extend to +/-2.7 times the standard deviation, and red crosses are outliers.

It is evident from the results of these experiments that exposure to high mean flow velocities can affect the health status of plants. Healthy leaves appear to become healthier when exposed to high mean flow velocities (Figure 13a). However, if one individual young mature leaf is used as indicator of plant health results can be biased as this leaf may not be representative of the whole plant. We recommend that plant health status is quantified by both values of  $F_v/F_m$  and assessment of plant foliage integrity; that is, leaves that are missing or are so decayed that they provide invalid measurements of  $F_v/F_m$  should be considered in the assessment of plant health. Users are therefore recommended to measure  $F_v/F_m$  on multiple leaves from each plant, and these leaves should be selected so that they are representative of the entire organism.

## 5.3 IMPACT OF HEALTH STATUS ON HYDRODYNAMIC PERFORMANCE OF *P. CRISPUS*

Flume experiments were conducted to establish if the health status of a plant can affect its hydrodynamics. A total of 27 plants of *P. crispus* were tested at three flow scenarios ('Run' 1, 'Run' 3, and 'Run' 5 in Table 6). Prior to experiments plants were exposed to different conditions (mainly



involving light and desiccation treatments) in such a way that they would show a broad range of health status. Preliminary results from two plants with high health status and two plants with low health status are shown here (Table 7). Plants with high health status looked healthy and had many green leaves, whereas plants with low health status were characterised by stems with few green leaves and were visibly less healthy. Each plant was selected from the storage tank, its health status was assessed by measuring  $F_v/F_m$  on the middle part of eight young mature leaves located within the top three internodes. Leaves were selected with the same criteria reported in the previous section (i.e. to be representative of the whole organism). The value of  $F_v/F_m$  was arbitrarily set to zero for missing leaves or leaves too deteriorated to provide valid measurements of chlorophyll fluorescence. The plant was then moved into the flume and attached to the bed where it was tested at flow scenario 'Run' 1, 'Run' 3, and 'Run' 5 (in sequential order) for 10 minutes each. At the end of the flume experiments plant health status was assessed using the same procedure applied in the previous experiments. The setup for flume experiments is shown in Figure 14a-b and included the use of two side-looking Vectrino+ ADVs (Nortek AS, Rud, Norway) to measure flow velocities 0.1 m upstream and downstream of the plant and use of a full HD camera to record plant position and movement in the longitudinal-vertical plane. The sampling frequency of ADVs was 60 Hz, the camera recorded at a 30 Hz with a resolution of 1920x1080 pixels (i.e. Full HD). The positions of ADV sampling volumes were adjusted so that the instruments would record at the flume centreline and at the same height as the centre of the plant. The hydrodynamic performance of each plant was assessed by analysing its effects on the flow velocities across a range of flow conditions and its static reconfiguration (using data extracted from side videos).

The effects of a plant on the flow characteristics were estimated as the variation in mean and standard deviation of the longitudinal velocity and turbulent kinetic energy due to the presence of the plant (i.e. comparing values upstream and downstream of it). For all plants mean longitudinal velocity decreased and turbulence was enhanced (i.e. turbulence intensity and turbulent kinetic energy increased) downstream of plants, and these effects tended to intensify as the mean flow velocity increased. However, there were no apparent differences in the effects of plants with low health status and plants with high health status (Table 7) on the flow characteristics in the single fixed-point measurements from the two ADVs.



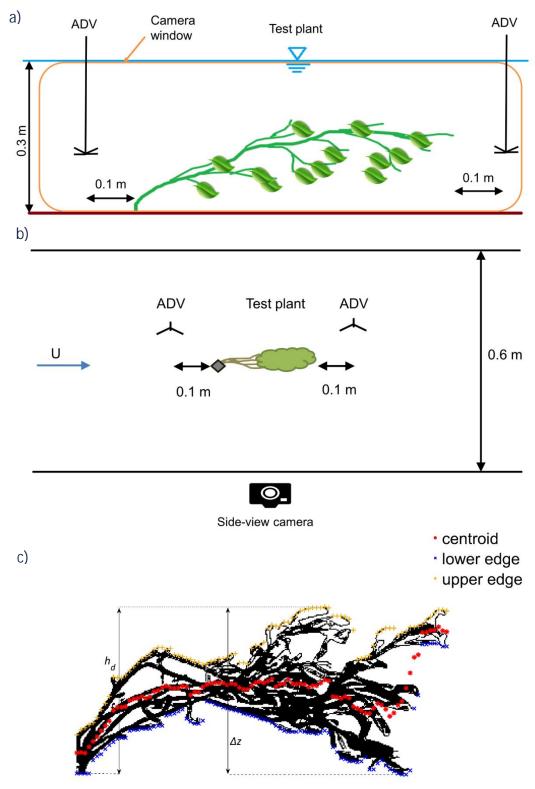


Figure 14 Sketch of the experimental setup: side view (a) and top view (b). (c) Example of data output from a video frame showing plant position (black), centroid, upper and lower edges, and definition of plant deflected height ( $h_d$ ) and frontal projected height ( $\Delta z$ ).

The hydrodynamic performance of a plant was assessed by estimating its ability to reconfigure at a range of mean flow velocities; that is, how its posture varied across the range of mean flow



velocities investigated. Plant reconfiguration was assessed using plant positions extracted from videos with a combination of edge detection algorithm and thresholding of RGB colour channels calibrated for each video. For purposes of analysis each video frame was divided into vertical regions 4-pixels wide, and for each of these regions the maximum, the minimum, and the mean vertical positions (also referred to as the centroid) of a plant were identified (Figure 14c). The timeseries thus obtained were then analysed to estimate the frontal projected height ( $\Delta z$ ) as the difference between the maximum and minimum vertical coordinates along a plant excluding the part of stem attached to the bed, and plant deflected height ( $h_d$ ) as the maximum vertical coordinate along a plant (Figure 14c). The time averaged frontal projected height ( $\Delta Z$ ) and plant deflected height ( $H_d$ ) were then calculated and normalized using plant length (D). Results for the four plants analysed are reported in Table 7. Further, the mean (D) and standard deviation (D) of centroid vertical position were calculated for each region. These parameters are displayed in Figure 15 normalized using plant length.

Plant $(\overline{F_v/F_m})$	Plant length - I (mm)	Normalized frontal projected height - ΔZ/I			Normalized plant deflected height - $H_d/I$		
		Run 1	Run 3	Run 5	Run 1	Run 3	Run 5
Plant 1 (0.29)	175	0.549	0.303	0.267	0.729	0.422	0.380
Plant 2 (0.19)	220	0.552	0.317	0.266	0.572	0.388	0.296
Plant 3 (0.74)	200	0.364	0.173	0.162	0.456	0.251	0.216
Plant 4 (0.74)	280	0.373	0.323	0.188	0.381	0.327	0.188

Table 7 Normalized time averaged frontal projected height and plant deflected height for plants at each flow scenario. The average maximum quantum efficiency  $(\overline{F_v}/F_m)$  of PSII is reported below plant identifier. Plant length is also reported.

The results for the frontal projected height and plant deflected height show that plants characterised by high health status deflected more than plants with low health status (Table 7). This indicates that healthier plants had a more efficient static reconfiguration and are expected to have better hydrodynamic performance (i.e. reduced drag). This is apparent in Figure 15 where the vertical position of centroids is higher for plants of lower health status. As expected, all plants became more prone as the flow velocity increased. It is, however, important to contextualize these preliminary results better by considering information on plant morphology, which is a primary factor in defining hydrodynamic performance. Plant health status was described here using the average (averaged across eight leaves) maximum quantum efficiency  $(\overline{F_v/F_m})$  of PSII and, therefore, accounting for the presence of leaves on plant stems. Plant 1 and 2 were characterised by low health status because some of their stems did not present any leaves in their top part. It follows that the different hydrodynamic performance of plants depending on their health status may be a consequence of foliage (i.e. a physical factor) rather than actual values of  $F_v/F_m$ .

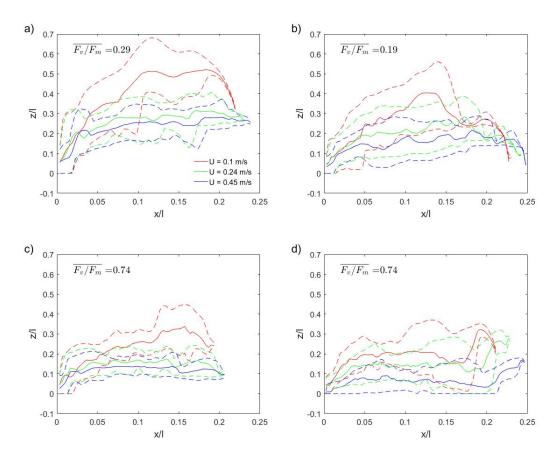


Figure 15 Vertical positions of plant centroids normalised using plant length (I) for plant 1 (a), plant 2 (b), plant 3 (c) and plant 4 (d). Solid lines represent mean position of centroids, dashed lines represent a range of twice the standard deviation around the mean (i.e.  $Z_c \pm \sigma_{zc}$ ). The average maximum quantum efficiency of PSII for each plant is reported on the top left corner of each plot.

In the cases investigated, single fixed-point measurements from two ADVs showed no significant differences in the impact on flow velocities and turbulence characteristics between healthy and unhealthy plants. It is likely that these results are affected by ADV sampling volumes being of limited size and therefore not able to characterise the spatially heterogeneous wakes downstream of plants. Furthermore, during experiments plants were observed to move sideways, and this motion is expected to have caused lateral displacements of the wakes so that ADV sampling volumes were temporarily located out of the wakes and ADVs did not record useful information on the effect of plants on the flow characteristics. We do not think this is a general result and expect that different measurements of flow velocities would yield differences in flow properties. Analysis of plant posture during experiments indicated that there are significant differences in reconfiguration of healthy and unhealthy plants. However, it is not clear whether this difference is mainly caused by foliage integrity rather than plant health status. Analysis of additional foliated plants (among those already tested) and further experiments including measurements of the drag force acting on plants are required to explore the relationship between plant health status and plant hydrodynamic performance.



## 6 HEALTH STATUS OF VEGETATION IN FLUME FACILITIES

#### 6.1 Conclusions

This deliverable identifies a simple and promising technique – chlorophyll fluorescence analysis – to monitor vegetation stress in flume facilities. A specific test, referred to as  $F_v/F_m$  test, is employed in several sets of experiments to assess the health status of freshwater macrophytes in a laboratory setting. These applications confirm that the technique is valuable for establishing vegetation health status. A protocol is designed so that practitioners with no biological background can use this technique in ecohydraulics applications.

Our findings indicate that the protocol can be applied to different types of vegetation with a range of leaf morphological traits. The selection of samples, their preparation and the measurement procedure are key for proper assessment of vegetation health status. In the cases investigated, sampled leaves should be located within the top three internodes of a stem and should be darkadapted for at least 15 minutes. Measurements should be taken in the central part of a leaf. It is essential that leaves are selected in such a way that they are representative of the entire organism used in the experiment, and total plant health is assessed by also considering deteriorated or missing leaves. Moreover, when designing flume experiments users should consider the growth cycle of the plant and the consequent effects of seasonality on plant health status, morphology and biomechanics.

Results of experiments in which the technique was applied indicate that laboratory conditions typical of hydraulic laboratories can affect vegetation health status and, in some cases, induce significant changes in vegetation mechanical properties. We expect there to be connections between plant physiology, biomechanics, and plant interactions with the flow across a gradient of responses. In the brief experiments we have conducted we have not explored these relationships in detail and our results are not conclusive. However, we have shown that chlorophyll fluorescence analysis is an important tool for quantifying plant health status and can be successfully applied to help us assess these issues.

### 6.2 ROADMAP

To explore the connections between plant physiology (e.g. health status), plant biomechanics, and flow-plant interactions in a comprehensive manner, we need to undertake systematic studies of these relationships investigating different types of vegetation and different stressors.

Even though the  $F_v/F_m$  test was successfully used to quantitatively assess the health status of freshwater macrophytes, our results show that  $F_v/F_m$  may not be able to detect stresses that occur at short temporal scales (e.g. hours). However, chlorophyll fluorescence and the devices we have recommended allow measurement of other quantities than  $F_v/F_m$  that may be more sensitive to specific stresses, including those which develop quickly. More work is required to establish the best protocols for different types of vegetation and the conditions to which they are exposed. To be able to assess plant stress from a wider range of sources, it may be necessary to apply a combination of techniques that monitor other activities or cycles within the organism including plant signalling networks (e.g. monitoring the concentration of proteins or metabolites in plant tissues).



Established tension and flexural tests can be used to measure a range of biomechanical properties of plant stems, but environmental factors are expected to have an effect on the biomechanics of leaves as well. The development of techniques that can measure the biomechanical properties of leaves would be beneficial. Also, the effect of vegetation on flow characteristics should be assessed using techniques that allow measurement of flow velocities on larger spatial scales (e.g. Particle Image Velocimetry) so that the entire wake can be characterised. Finally, the hydrodynamics performance of vegetation should be assessed using drag measurement devices and multiple cameras that allow resolving vegetation position and motion in the three dimensions.



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