The Use of Photosynthetic Fluorescence Parameters from Autotrophic Biofilms for Monitoring the Effect of Chemicals in River Ecosystems

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Abstract Photosynthetic processes play a key role in aquatic ecosystems. These processes are highly sensitive to the presence of toxicants, leading to an increase in their use as ecotoxicological endpoints. The use of chlorophyll-*a* fluorescence techniques to assess the impact of toxicants on the photosynthesis of the autotrophic component of fluvial biofilms has increased in the last decades. However, these photosynthetic endpoints are not currently used in water quality monitoring programs.

A review of the currently available literature—including studies dealing with toxicity assessment of both priority and emerging compounds—allowed the discussion of the pros and cons of their use as ecotoxicological endpoints in fluvial systems as well as their inclusion in regular monitoring programs.

Chlorophyll-*a* fluorescence measurements have the ability to detect effects of a large panel of chemical substances on the photosynthetic processes of fluvial biofilms, covering both functional and structural aspects of the biofilm community. Moreover, they might provide early warning signals of toxic effects.

Thus, the application of the chlorophyll-*a* fluorescence measurement is recommended as a complementary measurement of toxic stress in aquatic ecosystems.

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Their application is of special interest in the context of the Water Framework Directive (WFD, Directive 2000/60/EC), where the development of new structural and functional endpoints of the biological quality elements (e.g., biofilms) is required.

Keywords Biofilms • Chl-*a* fluorescence parameters • Emerging substances • Priority substances • Rivers

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Abbreviations

AL	Actinic light
BQE	Biological quality element
F	Fluorescence yield at the maximal reduced state
Fe	Ferrodoxin
Fm	Maximal fluorescence yield
Fm′	Fluorescence yield at actinic light steady state
Fo	Minimal fluorescence yield
Fo(Bl)	Fluorescence signal linked to cyanobacteria group
Fo(Br)	Fluorescence signal linked to diatoms’ algal group
Fo(Gr)	Fluorescence signal linked to green-algae algal group
Fo/Fv	Efficiency of the water-splitting apparatus of PSII
Fo′	Fluorescence yield when actinic light is omitted
Fv	Variable fluorescence yield
Fv/2	Fluorescence measurment of plastoquinone pool
ML	Measuring light
NPQ	Non-photochemical quenching without measuring Fo′
PAM	Pulse amplitude modulated
PEA	Plant efficiency analyzer
Pheo	Pheophytin
PQ	Plastoquinone pool
PQ_A	Plastoquinone A

PQ_B	Plastoquinone B
PS	Photosystem
PSI	Photosystem I
PSII	Photosystem II
qN	Non-photochemical quenching
qP	Photochemical quenching
SP	Saturation pulse
UQD_{rel}	Relative unquenched fluorescence
WFD	Water framework directive
Φ'_{PSII}	Effective quantum yield of PSII
Φ_{PSII}	Maximal quantum yield of PSII

1 Introduction

Photosynthesis is a major process for all illuminated ecosystems as it provides the main source of organic material for the food chain. Autotrophic organisms carry out this physiological process by converting the light energy into chemical energy, building up organic molecules out of CO_2 and water [1]. Derived organic matter serves as food of the heterotrophic organisms. Understanding the physiological/photochemical processes supporting this key function was focus of research for hundreds of years, and several measuring techniques were developed based on chemical analysis of pigments, ^{14}C fixation, oxygen production, or chl-*a* fluorescence-based methods, among others. The latter methods provide a good basis for application in monitoring algal density or photosynthetic processes in terms of electron transport activity and energy dissipation processes [2].

The use of chl-*a* fluorescence techniques to assess photosynthesis performance under changing environmental conditions has widely been proved as a rapid, noninvasive, reliable method [3, 4]. Since the first acknowledgement of the analytical potential of chl-a fluorescence techniques [5, 6] until now, extensive research has been carried out to apply this technique in different research fields. In the case of ecotoxicology, chl-*a* fluorescence techniques have been used to evaluate the toxicity of different pollutants and to locate their primary sites of damage in photosynthetic organisms.

This review focuses on the use of chl-*a* fluorescence techniques to assess chemical effects on the autotrophic component of fluvial biofilms, a nontarget community present in all aquatic systems. Fluvial biofilms are made up mainly of algae, bacteria, fungi, and other micro- and meiofauna organisms, embedded in a matrix of extracellular polymeric substances [7]. In contrast to other autotrophic groups, such as phytoplankton, biofilms have the particularity to live immobile; therefore they are suitable for long-term monitoring and allow chemical toxicity to be assessed at community level. This community approach is much closer to the processes of an ecosystem than the use of single species tests and uses a biological quality element (BQE) [8, 9], which is regularly monitored within the Water Framework Directive (WFD, Directive 2000/60/EC) for the assessment of the

ecological status of water systems. Biofilms have the capacity to modify the transport and accumulation of substances such as nutrients [10] as well as organic toxicants and heavy metals [11]. Several studies have highlighted the sensitivity of these communities to a large panel of toxicants such as heavy metals [12–14], herbicides [15–19], pharmaceuticals, and personal care products [20–23]. Due to this sensitivity, fluvial biofilms can be used as early warning systems for the detection of the effects of toxicants on aquatic systems [24]. The pertinence of the use of chl-*a* fluorescence techniques for toxicity assessment on fluvial biofilms is attributed to the basis that if a chemical produces effects on the photosynthesis processes (in a direct or indirect way) the chl-*a* fluorescence parameters will reflect it. In the last decade several investigations followed this approach [12, 15, 18, 20, 25–31, 33].

Based on the chl-*a* fluorescence techniques mainly two types of analysis of fluorescence have been developed to assess photosynthesis performance: (a) the fast fluorescence induction kinetics, measured by plant efficiency analyzer (PEA) instruments, and (b) the slow fluorescence induction kinetics, measured by pulse amplitude modulated (PAM) fluorometers. Although the first provides interesting information on various steps of photosynthetic electron transport [32], it has not been applied to complex samples such as fluvial biofilms.

Several PAM fluorescence instruments are provided to assess the photosynthetic performance in biofilms; each one presenting a specific characteristic: (1) Standard PAM fluorometers excite chlorophyll fluorescence at one wavelength (665 nm, excitation maximum of the chlorophyll-*a* molecule) and have been applied on biofilms to evaluate the global photosynthetic response of the autotrophic compartment (e.g., [15, 25]). PAM fluorometers are available in different technical settings (Fa. Walz, Effeltrich, Germany). Next to standard applications working with cuvettes, a microscopy PAM fluorometer is available, suitable for the assessment of selected cells within a biofilm on a microscopic scale [33]. The Maxi-Imaging PAM fluorometer was developed to assess the photosynthetic capacity of large surfaces, e.g., leaves or multiwell plates and is also suitable for measuring biofilms [34, 35]. (2) Multiwavelength excitation PAM fluorometers (e.g., Phyto-PAM) present the singularity to work with several excitation wavelengths, exciting pigments with different absorption spectra, e.g., carotenoids, which are characteristic for defined algal classes. After deconvolution of the fluorescent signals from mixed algal samples the multiwavelength PAM fluorometry has the potential to reveal the contribution of algal groups with different absorption spectra [36].

Different sources of chemical contamination (industrial, agricultural, or urban activities) discharge a wide variety of compounds with different modes of action (MoA), toxic concentrations, persistence into the ecosystem (accumulation, degradation), etc.

The WFD (2000/60/EC) defines a strategy for protecting and restoring clean waters across Europe. As a first step of this strategy, a list of priority substances was adopted in the Directive 2008/105/EC, in which 33 substances of priority concern were identified and regulated. The list includes mainly organic contaminants, such as pesticides, and four toxic metals [37]. Besides this recognized contaminants, more substances are being detected in the environment. The so-called emerging

contaminants are compounds that are not currently covered by existing water quality regulations and are thought to be potential threats to environmental ecosystems [38]. The WFD follows two different assessment strategies: the chemical status evaluation and the ecological status. The "ecological status" represents the "quality of the structure and functioning of aquatic ecosystems associated with surface waters" (Directive 2000/60/EC). In order to establish the "ecological status," the WFD requires the sampling and interpretation of data on a broad suite of "BQEs." The WFD required that observed metric values for BQEs in a water body undergoing monitoring were mathematically compared with expected values for reference condition sites based on predictive modeling, hind casting, or expert judgment [39]. Biofilms, referred to as periphyton in the WFD, have been included as one major BQE. Concerning this BQE, the WFD recognized the diatom index within biofilms to evaluate structural effects, which has been widely applied for water managers. For non-diatom species from periphyton there is not a recognized parameter to be evaluated.

This chapter introduces the measuring principles of chl-*a* fluorescence techniques and summarizes studies that assess the toxicity of priority and emerging substances on autotrophic biofilms by using chl-*a* fluorescence techniques. The photosynthesis mechanism has been shown to be very sensitive to several toxic substances such as heavy metals or herbicides [40, 41]. So the photosynthesis process which is essential for the overall survival of phototrophs can be used as an ecotoxicological endpoint to assess the impact of many toxicants [42].

The main aim of this chapter is to analyze the pros and cons of the use of chl-*a* fluorescence techniques for water quality monitoring programs in the context of the WFD. While not being an exhaustive review, 29 different investigations have been analysed, including only field and laboratory investigations dealing about the application of chl-a fluorescence parameters on biofilm communities.

2 Physiological Basis of Photosynthesis

Photoautotrophic organisms have the ability to synthesize organic compounds from CO_2 and water by converting light energy to molecular energy during photosynthesis. Oxygenic photosynthesis is catalyzed in two photosystems (PS): PSI and PSII containing chlorophyll molecules that are embedded in the thylakoid membrane of the chloroplasts as integral membrane protein complexes. Starting point of photosynthesis is the absorption of photons by the pigment molecules (chl *a*, chl *b*, phycobiliproteins, and carotenoids) of the antennae systems and the transfer of energy of excited molecules to the reaction centers of PSI and PSII (Fig. 1). The central chlorophyll molecule of the PSII, the first of the two photosystems activated during this process, is excited by a previously excited molecule, in a type of energy transfer that is called resonance energy transfer or excitation transfer. Promoted by a Mn complex (an enzyme complex), the reaction center of the PSII gets electrons from the cleavage of a water molecule, a reaction that produces O_2 and protons H^+.

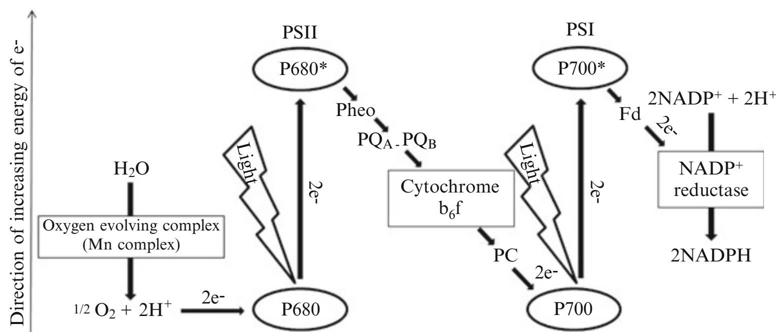

Fig. 1 Schematic overview of the *z*-scheme of electron transport between the two photosystems (PSII and PSI) in the photosynthesis processes

The electrons from the water cleavage are subsequently transferred by a complex system of acceptor and donor molecules: pheophytin (Pheo), plastoquinone A (PQ_A) and B (PQ_B), the plastoquinone pool (PQ), and the cytochrome complex to the PSI (Fig. 1). Finally, the excited molecules are transferred from the PSI to ferredoxin (Fd), another acceptor molecule, which catalyzes the reduction of $NADP^+$, a phosphorylated derivate that carries reducing electrons. These energy-rich products from photosynthesis are later used in the Calvin cycle to build up hexoses and other organic matter [1]. The chl-*a* fluorescence techniques allow to evaluate different photosynthetic processes occurring under light excitation.

3 Chl-*a* Fluorescence Analysis and Derived Parameters

Two types of chl-*a* fluorescence analysis have been developed and applied in ecotoxicological studies: the "fast fluorescence induction kinetics" [32] and the "slow fluorescence induction kinetics" [43].

3.1 The Fast Fluorescence Induction Kinetics: PEA Fluorometry

The rapid rise of fluorescence is measured with PEA and provides information on various steps of the photosynthetic electron transport [32]. In the fast fluorescence induction kinetics, the chl-*a* fluorescence transient follows a polyphasic pattern of O–J–I–P electrons transients from the initial fluorescence level (Fo or O) to the maximum fluorescence level (Fm or P) (Fig. 2). The rise from O (at 0.05 ms) to the J phase (at 2 ms) is due to the net photochemical reduction of plastoquinone (PQ_A–PQ_A) (photochemical phase). The J–I phase (at 30 ms) is due to the closure of the remaining photosynthetic centers, and the I–P (ends about at 500 ms) is due to the removal of plastoquinone quenching due to the reduction of PQ (non-photochemical phase) [32]. The decrease in Fm and fluorescence levels at phases

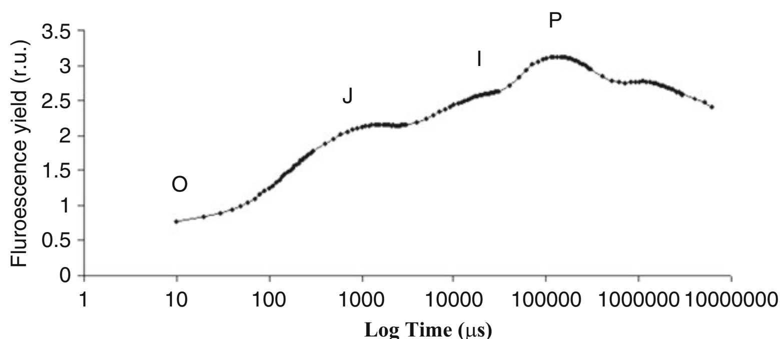

Fig. 2 Typical rapid fluorescence kinetics measured with PEA fluorometer

J and I is normally explained by the inhibition of the electron transport at the electron donor side of PSII, which results in the accumulation of the excited reaction center of PS II (P680*), a strong fluorescence quencher [44]. Thus the fluorescence rise provides information on various steps of photosynthetic electron transport [32]. The kinetics of OJIP transients obtained allows evaluating the toxic effect of chemicals on specific characteristics of PSII as energy trapping processes or antenna size. Besides, by using this fast fluorescence kinetics it is also possible to calculate different photosynthetic parameters and the complementary area (CA) [45]. The CA is a measure of the kinetics of fluorescence induction up to the P level and it has been reported as a direct indicator of the PSII photochemistry [46]. CA has been used for many years to assess the phytotoxicity of pollutants [40]. It is reported that fast fluorescence induction kinetics can also allow the location of the primary site of damage induced by environmental stress [47].

3.2 The Slow Fluorescence Induction Kinetics: Standard PAM Fluorometry

The slow fluorescence induction analyses are carried out by using PAM fluorometry. The so-called saturation pulse quenching analysis based on the principle that light energy absorbed by PSII pigments can drive the photochemical energy conversion at PSII reaction centers, be dissipated into heat, or be emitted in the form of chl *a* fluorescence. As these three pathways of energy conversion are complementary, the fluorescence yield may serve as a convenient indicator of time- and state-dependent changes in the relative rates of photosynthesis and heat dissipation. The PAM fluorescence method employs a combination of three different types of light: modulated or measuring light (ML) = 0.05 μmols photons m^{-2} s^{-1} in μsec pulses; actinic light (AL) = from 1 to 600 μmols photons m^{-2} s^{-1}; and strong saturation pulses (SP) = 8,000 μmols photons m^{-2} s^{-1} (Fig. 3), which allow the adequate analysis of the fluorescence induction kinetics of photosynthetic organisms.

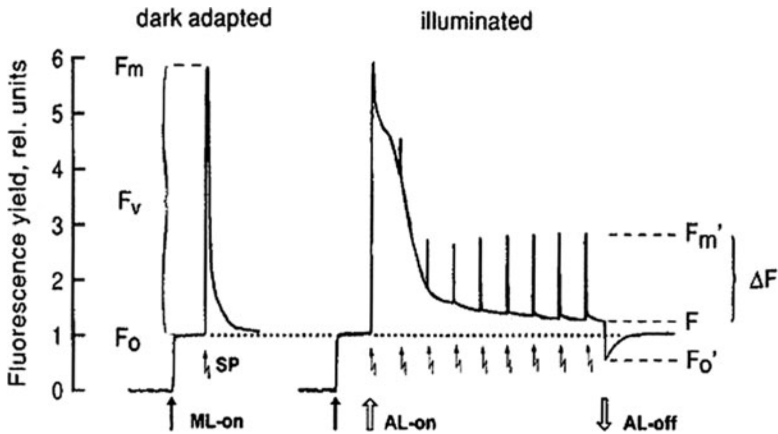

Fig. 3 Schematic representation of the slow fluorescence kinetics analysis by using the PAM fluorometry. The different types of light are indicated (*ML* measuring light, *AL* actinic light, *SP* saturation pulse). Modified from Schreiber [89]

By PAM fluorometry we can get two different types of measures: a biomass-related ones (based on basal fluorescence emission) and a functional ones (based on changes in fluorescence emission caused by strong saturation pulses).

During PAM fluorescence analysis, different information can be obtained from a dark- or light-adapted sample.

3.2.1 Chlorophyll Fluorescence Characteristics in the Dark-Adapted State

In a dark-adapted phototropic organism modulated light with low energy is unable to trigger electron transport; then the small fluorescence yield corresponds to the constant fluorescence (Fo), which represents the light dissipation by excited antennae of Chl-*a* molecules before the excitation energy is transferred to the reaction centers of PSII [48–50].

The maximal fluorescence yield (Fm) is observed, when all PSII reaction centers are closed after the application of a strong saturation pulse. The difference between Fm and Fo is the maximal variable fluorescence yield (Fv). Fv/Fm is used as a measure of the maximal photochemical efficiency of PSII (Fig. 3).

3.2.2 Chlorophyll Fluorescence Characteristics in the Light-Adapted State

Actinic light maintains the photosynthetic process in an active state, providing the appropriate conditions to analyze fluorescence kinetics. The maximum fluorescence yield, reached under this condition, will determine the fluorescence transient (F), where electron transport carriers are at a maximum reduced state. The

maximum variable fluorescence induced by a set of saturating flashes at a steady state of fluorescence is represented by Fm′. The difference between Fm′ and F indicates the variable fluorescence induced by actinic light at a steady state of electron transport (ΔF). The Fo′ is the constant fluorescence when actinic light is omitted (Fig. 3).

3.2.3 Parameters Used and Their Interpretation

Deriving from these dark and light chl-*a* fluorescence measurements several parameters can be obtained (Table 1) and applied in ecotoxicological studies, to assess the "health" status of the photosynthetic organisms through photosynthetic activity measurements.

The *minimal fluorescence yield (Fo)* reflects the chl-*a* fluorescence emission of all open reaction centers in a non-excited status. Fo can be used as a surrogate of algal biomass since chlorophyll fluorescence is proportional to total chlorophyll content [51, 52]. It is expected that Fo will decrease if toxic exposure causes a reduction in the number of cells due to cell death (structural damage) or the chlorophyll content of a sample. Biomass estimations based on Fo are not always possible, losing linearity above a given biomass level if the thickness of the biofilms is excessive producing self-shading [15, 53].

Table 1 Fluorescence parameters obtained by using PAM fluorometry

Parameter	Name	Equation	References
Φ_{PSII}	Maximal quantum yield	$\Phi_{PSII} = (Fm - Fo)/Fm$	Schreiber et al. [49] and Genty et al. [54]
Φ'_{PSII}	Effective quantum yield	$\Phi'_{PSII} = (Fm' - F)/Fm'$	Schreiber et al. [49] and Genty et al. [54]
qP	Photochemical quenching	$qP = (Fm' - F)/(Fm' - Fo')$	Schreiber et al. [49], Horton et al. [57], and Müller et al. [90]
qN	Non-photochemical quenching	$qN = 1 - [(Fm' - Fo')/(Fm - Fo)$	Schreiber et al. [49], Horton et al. [94], and Müller et al. [90]
NPQ	Non-photochemical quenching	$NPQ = (Fm - Fm')/Fm'$	Bilger and Björkman [91]
UQF_{rel}	Relative unquenched fluorescence	$UQF_{rel} = (F - Fo')/(Fm' - Fo')$	Juneau et al. [60]
Fv/2	Plastoquinone pool	$Fv/2 = (Fm - Fo)/2$	Bolhàr-Nordenkampt and Öquist [92]
Fo/Fv	Efficiency of the water-splitting apparatus of PSII	$Fo/Fv = Fo/(Fm - Fo)$	Kriedemann et al. [93]
Fo	Minimal fluorescence yield		Serôdio et al. [52] and Rysgaard et al. [51]

The *maximal or optimal quantum yield (Φ_{PSII})* measures the quantum yield of PSII electron transport in a dark-adapted state (Table 1). It has been shown in many studies that Φ_{PSII} can also be a measure of the quantum yield of photosynthesis [4, 54]. It is an estimate of the potential maximal photosynthetic activity and it is expected to be effected if a chemical produces alterations in the structure of the photosynthetic apparatus (e.g., shade-adapted chloroplasts); in general this situation occurs when biofilms are exposed to chemicals at high concentrations or during a long-term exposure. The Φ_{PSII} parameter could be named with other nomenclatures or names in the bibliography: Φ_o [55], Fv/Fmax [18], photosynthetic capacity [20], or photosynthetic activity [21]. In order to standardize nomenclatures we recommend the use of Φ_{PSII}.

The *effective or operation quantum yield (Φ'_{PSII})* measures the efficiency of excitation energy capture by the open PSII reaction centers under light conditions [49, 54], proportional to photosynthetic efficiency. A reduction of the Φ'_{PSII} indicates that the toxicant is reducing electron flow in the PSII. This parameter is very sensitive to PSII-inhibiting herbicides, if they block the electron transport flow (Table 1). The Φ'_{PSII} parameter could be named with other nomenclatures or names in the bibliography: Φ_{II} [25], Φ_{PSII} [56], yield or Y [28], photon yield [26], photosynthetic efficiency at PSII or Yeff [29], or Yield II [53]. In order to standardize nomenclatures the use of Φ'_{PSII} is recommended.

The *photochemical quenching (qP)*, which is determined by the redox state of Q_A, the primary electron acceptor of PSII [4], represents the proportion of excitation energy "trapped" by open PSII reaction centers used for electron transport [57] (Table 1).

The *non-photochemical quenching (qN)* reflects the amount of light energy dissipation inducing fluorescence quenching that involves nonradiative energy process [57] (Table 1).

The *non-photochemical quenching without measuring Fo′ (NPQ)* is a simplified non-photochemical quenching value, which assumed that NPQ is caused only by one quenching factor [58], omitting other energy-consuming processes, not directly involved in the PSII activity [59] (Table 1).

The *relative unquenched fluorescence (UQD_{rel})* is a parameter proposed by Juneau et al. [60]. It is complementary to the relative quenching components qP_{rel} and qN_{rel} proposed by Buschmann [58] that take into account the fraction of the non-quenched fluorescence yield related to the proportion of closed PSI reactive centers present under continuous irradiation (Table 1).

The *plastoquinone pool (Fv/2)* is a measure of the state of the pool of plastoquinones. Its reduction was linked with inhibitory effects of metals on the photosynthetic efficiency [61] (Table 1).

The *efficiency of the water-splitting apparatus of PSII (Fo/Fv)* reflects the state of water-splitting this complex. Metal exposure may damage this apparatus [61] (Table 1).

3.2.4 Multiwavelengths PAM Fluorometry

The PAM fluorescence methodology is now available and applied in several technical settings to monitor the influence of stress factors on microalgae photosynthesis (e.g., [95, 33, 36, 62]). In principle, this technique allows the researcher to address different levels of biological complexity using the same fluorescence parameters and was used in a comparative study by Schmitt-Jansen and Altenburger [33]. For uniform suspensions of unicellular algae measurement of variable chl-*a* fluorescence is a relatively straightforward exercise. Schreiber et al. [36] used an ultrasensitive dual-channel chlorophyll fluorometer for the assessment of diuron, deriving detection limits, sufficient for the demands of the European Commission drinking water regulation (0.1 μg/L for each individual substance).

Observation of variable fluorescence in biofilm communities, however, requires distinction between the contributions from the different algal components with respect to their differences in pigmentation and photosynthetic properties. Recently, a microscopic setup became available enabling PAM chl-*a* fluorescence measurements at a microscopic scale that allows excitation and detection of fluorescence from single algal cells [95]. Therefore, the noninvasive assessment of individual stress responses of cells in biofilms became possible. Another way to address this challenge is to simultaneously use excitation light of different wavelengths for excitation of the algal class-specific light harvesting complexes. For instance, Chl *b*, which characterizes chlorophytes as a key pigment, shows absorption peaks at 470 and 645 nm. Diatoms which are characterized by the key pigments Chl *c* and carotenoids, especially fucoxanthin, can be studied by fluorescence excitation in the blue and green spectral range (400–665 nm). On the other hand, in the case of cyanobacteria, fluorescence excitation in the blue and green is weak, while strong excitation is observed around 620–640 nm, in the absorption range of phycocyanin/allophycocyanin.

Using a four-wavelength excitation method, a differentiation of photosynthetic parameters of microalgal communities between different spectral groups, e.g., chlorophytes, cyanophytes, and diatoms, seems possible [62]. This application employs an array of light-emitting diodes (LED) to excite chlorophyll fluorescence at different wavelengths (470, 520, 645, and 665 nm). This particularity gives the opportunity to deconvolute the overall fluorescence signal into the contributions of algal groups [cyanobacteria-Fo(Bl), diatoms-Fo(Br), and green algae-Fo(Gr)] from community samples, e.g., phytoplankton or biofilms, based on the internal "reference excitation spectra" of a pure culture [36]. This application has been validated in fluvial biofilms by Schmitt-Jansen and Altenburger [53]. Besides the provision of a relative measure of the fluorescence abundance of each algal group it is possible to obtain values from fluorescence parameters specific of each phototrophic group (green algae, diatoms, or cyanobacteria) present in the community.

Beutler et al. [63] successfully applied a five-wavelength fluorometer to characterize a depth profile of the plankton algal community of Lake Plußsee, Germany.

4 The Use of Fluorescence Parameters to Assess the Effects of Toxicants on Biofilms

Chl-*a* fluorescence techniques based on PAM fluorometry have commonly been used to assess the effects of chemicals on biofilms. More precisely, photosystems I and II were recognized to be a good target for pesticides to control weed in agriculture; therefore several herbicides were developed, blocking photosystems, like the triazines or the phenylurea herbicides. The usefulness of fluorescence parameters derived after multiturnover flashes has been demonstrated for herbicides specifically inhibiting PSII activity in several studies [18, 19, 64–66]. On the other hand, fast fluorescence techniques measured by plant efficient analysis (PEA) have never been applied to assess chemical toxicity on biofilms. However, its use on algal monocultures is well reported [40, 45, 67, 68]. The information provided by PEA flurometers is different to that obtained by PAM fluorometry; the first allows evaluating the toxic effects on PSII energy trapping and PSII antenna size. For instance, Dewez et al. [68] assessed Cu and fludioxonil effects on *Selenastrum obliquus* by using PAM and PEA photosynthetic methods as well as by evaluating different antioxidant enzymatic activities. They observed that Cu may alter the energy storage in algae during photosynthesis but fludioxonil inhibitory effect appeared not to be directly associated with photosynthetic electron transport as seen for copper inhibition. By using the PEA method it was observed that the fluorescence yields at J, I, and P transients were quenched under Cu exposure (1–3 mg/L) and the authors attributed this disturbance on the energy trapping to an inhibition of the PSII electrons transport via PQ_A, PQ_B, and the plastoquinone pool. Also, they observed that copper altered the energy dissipation via the non-photochemical energy dissipation (by PAM method) and the change of the structural organization of the antenna size via PSII light harvesting complex (by PEA method). So, by using PEA and PAM fluorometers different but complementary information was obtained to evaluate deeply Cu toxicity on photosynthesis processes of *S. obliquus*.

Several studies are presented in order to illustrate the pros and cons of the application of PAM fluorometry technique on biofilm communities' growth in the laboratory to assess chemical toxicity of compounds with different MoA: PSII inhibitors (Sect. 4.1), other photosynthetic inhibitors (Sect. 4.2), and toxicants with unknown MoA on algae (Sect. 4.3). The use of PAM fluorometry to assess chemical toxicity in field studies is presented in Sect. 4.4. PAM fluorometry gives the opportunity to evaluate both functional and structural alterations in autotrophic organisms. These alterations are related with the MoA of the toxicants as well as the dose and the time of exposure.

4.1 PSII Inhibitors

Pollutants with MoA directly interfering with the PSII are mainly represented by organic herbicides (e.g., diuron, atrazine). Due to their specific MoA, studies dealing with their toxicity commonly use photosynthetic endpoints. The inhibition of the maximal and the effective quantum yield (Φ_{PSII}, Φ'_{PSII}) in biofilms exposed to PSII inhibitors for short periods of time has been already demonstrated. McClellan et al. [34] detected a reduction of the Φ_{PSII} in biofilms exposed to diuron (Table 2). Similar results have been obtained with atrazine, prometryn, and isoproturon ([19, 33, 53]; see Table 2 for detailed information). The sensitivity of biofilms to isoproturon under different light intensities was determined by Laviale et al. [69], showing a clear reduction of photosynthesis (Φ'_{PSII} and Φ_{PSII}), and, under dynamic light conditions, a clear reduction of the non-photochemical quenching (NPQ) mechanisms was observed (Table 2). Effects of PSII inhibitors on the biofilm structure are less reported, especially in the short-term toxicity assessment. Schmitt-Jansen and Altenburger [18] found a 90% reduction of algal biomass after 1 h of exposure to isoproturon (Table 2).

The chl-*a* fluorescence methods have also been applied to long-term toxicity studies. Ricart et al. [29] analyzed the long-term effect of the herbicide diuron on fluvial biofilms and detected a clear decrease of the Φ'_{PSII} effective quantum (Table 2). Ricart et al. [29] analyzed the long-term effect of the herbicide diuron on fluvial biofilms and detected a clear decrease of the effective quantum (Table 2) with increasing concentrations of diuron (Fig. 4), indicating a clear dose-dependent effect, probably attributable to the MoA of the herbicide. A reduction of the Φ_{PSII} of biofilms was also observed by Tlili et al. [70] after 3 weeks of exposure to diuron (Table 2). Schmitt-Jansen and Altenburger [18, 53] used these techniques to evaluate the long-term effects of isoproturon to biofilm structure. They detected a reduction in the algal biomass (measured as Fo) and a shift of the algal classes to a dominance of green algae (Table 2). Effects of diuron on a simple food chain (biofilm and grazers (snail *Physella [Costatella] acuta*)) have also been evaluated using PAM fluorometry techniques. In a long-term experiment, López-Doval et al. [71] used Φ'_{PSII} to monitor the physiological state of the biofilm, as well as to detect the effect of diuron, grazers, and their interaction on the biofilm community. A significant reduction in Φ'_{PSII} was detected in both diuron and diuron + grazers microcosms.

Chl-*a* fluorescence parameters have been used as a physiological endpoint to investigate community adaptation following the pollution-induced community tolerance (PICT) approach [72]. Briefly, the replacement of sensitive species by tolerant ones driven by toxicant's selection pressure is expected to increase the EC_{50} of the community measured in acute dose–response tests using physiological endpoints such as Φ'_{PSII}. PICT of periphyton to isoproturon [18, 53], atrazine, prometryn [19], and diuron [70] has been determined by testing inhibition of Φ'_{PSII} on pre-exposed and non-pre-exposed biofilm communities.

Table 2 Summary of studies applying chl-*a* fluorescence techniques to assess the toxicity of PSII inhibitors on fluvial biofilm communities

Toxicant	Biofilm community	Exposure	Conc.	Φ_{PSII}	Φ'_{PSII}	NPQ	Fo	Fo(Bl)	Fo(Gr)	Fo(Br)	Reference
Diuron	Microcosms	Short-term	0.4–100 μg/L		– – –						McClellan et al. [34]
Diuron	Microcosms	29 days	0.07–7 μg/L		– – –						Ricart et al. [29]
Diuron	Microcosms	3 weeks	10 μg/L	– –							Tlili et al. [70]
			10 μg/L + PO_4^{3-}	n.s.							
Diuron	Microcosms	29 days	2 μg/L		– –						López-Doval et al. [71]
Atrazine	Microcosms	1 h	7.5–2,000 μg/L		– – –						Schmitt-Jansen and Altenburger [19]
Prometryn			5–5,110 μg/L		– – –						
Isoproturon			2.4–3,120 μg/L		– – –						
Isoproturon	Microcosms	1 h	2.4–312 μg/L		– – –				+	– – –	Schmitt-Jansen and Altenburger [18]
Atrazine	Microcosms	1 h	7.5–2,000 μg/L		– – –						Schmitt-Jansen and Altenburger [33]
Isoproturon		1 h	2.4–312 μg/L		– – –						
Atrazine	Microcosms	1 h	7.5–2,000 μg/L		– – –						Schmitt-Jansen and Altenburger [53]
Prometryn			2.5–320 μg/L		– – –						
Isoproturon			2.4–312 μg/L		– – –						
Isoproturon	Microcosms	26 days	2.4–156 μg/L				– –		+ +	– –	
Isoproturon	Microcosms (constant light)	7 h	0–2,000 μg/L	– –	– – –	n.s.					Laviale et al. [69]
	Microscosms (dynamic light)	7 h	2, 6 and 20 μg/L	– –	– – –	+ + +					

The significant responses observed are indicated by "–" for inhibition or by "+" for increase and its approximate magnitude by: "–" or "+" if it was slight (10–30% of control), by: "– –" or "+ +" if it was moderate (30–50% of control) and by "– – –" or "+ + +" if it was high (>50% of control)
n.s. not significant ($p > 0.05$)

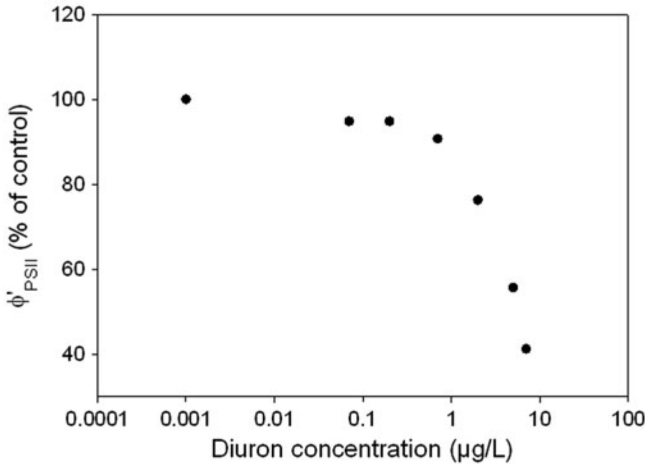

Fig. 4 Diuron effects on effective quantum yield of biofilm communities exposed to increasing concentrations of the herbicide. Modified from Ricart et al. [29]

4.2 Other Photosynthetic Inhibitors

Chl-*a* fluorescence parameters have commonly been used to assess the toxicity of chemicals affecting other processes than PSII electron transport. In these cases, the obtained parameters may indicate secondary effects on the performance of the photosynthetic apparatus or on algal biomass. For instance copper is a phytotoxic chemical affecting the activity of photosystem II and electron transfer rates [61]. However, this metal is also known to cause the oxidation of sulphydryl groups of enzymes leading to their inhibition [73] or to generate reactive oxygen species like superoxide and hydroxyl radicals causing oxidative stress (for a review see [14]) reducing growth as well as photosynthetic and respiratory activities [74]. Several biofilm studies have used copper as model toxic compound (Table 3), applying in many cases chl-*a* fluorescence parameters to assess biological responses. Serra et al. [28] measured Φ'_{PSII} to check the photosynthetic performance of biofilms during the Cu retention experiment that lasted for few hours. Transient inhibition of Φ'_{PSII} was also observed by Corcoll et al. [31] after few hours of Zn exposure; however this parameter recovered after longer exposure (Table 3).

Physiological chl-*a* parameters such as Φ'_{PSII} may not consistently show the effects caused by non-PSII inhibitors after longer exposure due to community adaptation. Barranguet et al. [56] observed that biofilm exposed during 2 weeks to Cu presented alterations in the Fo and that Φ'_{PSII} was less affected. Barranguet et al. [25] observed that the main factor regulating the sensitivity of biofilms to Cu toxicity (based on the Φ'_{PSII} endpoint) during short-term exposures was the physical structure of the biofilm (package of cells and thickness), and not to the species composition. These endpoints were used to show differences in sensitivity between suspended cells and biofilm communities. Fo and Φ'_{PSII} were also used

Table 3 Summary of studies applying chl-*a* fluorescence techniques to assess toxicity on fluvial biofilm communities of compounds with other effects on photosynthesis (different from PSII inhibition)

Toxicant	Biofilm community	Exposure	Conc.	Φ_{PSII}	Φ'_{PSII}	NPQ	Fo	Fo(Bl)	Fo(Gr)	Fo(Br)	References
Cu	Microcosms	24 h; 3 days	445–1,905; 126 μg/L		−/− −		− − −				Barranguet et al. [25, 56]
Zn	Microcosms	72 h	320 μg/L	n.s.	−	n.s	n.s.	n.s.	n.s.	n.s.	Corcoll et al. [31]
		5 weeks	320 μg/L	−	n.s.	− − −	− − −	n.s.	n.s.	−	
Cu	Microcosms	6; 16 days	0–100 μg/L		−		− − −				Guasch et al. [12, 27]
Cu, Zn, Cu + Zn	Microcosms	5 days	636; 6,356 μg/L	−	n.s.			− − −	− − −		García-Meza et al. [55]
Cu	Microcosms	4 weeks	30 μg/L					+ + +	n.s.	− − −	Serra et al. [28]
	no Cu vs. Cu pre-exposure	6 h	100 μg/L		−/n.s.		n.s				
Cu	Microcosms	3 weeks	30 μg/L	−							Tlili et al. [70]
			30 μg/L + PO_4^{3-}	n.s.							

Significant responses observed are indicated by "−" for inhibition or by "+" for increase and its approximate magnitude by "−" or "+" if it was slight (10–30% of control), by "− −" or "+ +" if it was moderate (30–50% of control) and by "− − −" or "+ + +" if it was high (>50% of control)
n.s. not significant ($p > 0.05$)

by Guasch et al. [12] and [55] to assess Cu and Cu plus Zn toxicity, respectively. In both cases Fo appeared to be the most sensitive endpoint, whereas effective concentrations based on Φ'_{PSII} were always much higher (Table 3). In the second example, multiwavelength fluorometers allowed following effects on the biomass of the cyanobacteria and green-algae groups of the biofilm as well as their respective Φ_{PSII}. Similarly, Serra et al. [28] demonstrated that community composition differed among biofilms with different Cu-exposure history. Community changes were identified using multiwavelength fluorometry, whereas Cu adaptation was assessed by comparing the Φ'_{PSII} responses to short Cu exposure (Table 3). Tlili et al. [70] used the Φ_{PSII} to evaluate the role of phosphorus on Cu toxicity on biofilms. They observed that biofilms pre-exposed to Cu presented a lower Φ_{PSII} than biofilms non-pre-exposed to Cu or pre-exposed to Cu plus phosphorus (Table 3).

4.3 Toxicants with Unknown Mode of Action on Algae

The impacts of personal care products and pharmaceuticals on the environment are not generally well known [75] and its effects on fluvial biofilms have poorly been investigated. Chemicals that damage membranes or proteins associated with photosynthetic electron transport or that inhibit any cellular process downstream of PSII, such as carbon assimilation or respiration, will lead to excitation pressure on PSII [76]. Therefore, chl-*a* fluorescence methods can also be used to assess the effects of chemicals affecting metabolic processes not directly linked to photosynthetic electron transport. This approach has recently been used to provide complementary endpoints to evaluate direct and indirect effects of these compounds on biofilm [20, 21, 23].

The acute toxicity of three β-blockers: metoprolol, propranolol, and atenolol on fluvial biofilms was assessed by using several biomarkers, including chl-*a* fluorescence parameters measured with a multiwavelength PAM to evaluate toxic effects on the autotrophic biofilm compartments (Fig. 5) [20]. They observed that propranolol was the most toxic for algae, causing 85% inhibition of Φ'_{PSII} (photosynthetic efficiency). Metoprolol was particularly toxic for bacteria and atenolol affected similarly bacterial and algal compartments of the biofilm but to a lesser extent (Table 4). Moreover, the use of chl-*a* fluorescence parameters for three algal groups gave an interesting insight into the algal compartment (Fig. 5). The relative contribution of the different algal groups was also used to obtain the effective photosynthetic efficiency for each of them, using the fluorescence signal linked to green algae, cyanobacteria, and diatoms. This approach allowed the detection of the earlier sensitivity of cyanobacteria-Fo(Bl) compared to green-Fo(Gr) and brown algae-Fo(Br) to propranolol. On the other hand, atenolol affected green algae and cyanobacteria photosynthetic efficiencies while diatoms seemed resistant to this toxic compound. Consequently, they concluded that the chl-*a* fluorescence parameters are powerful tools within a multibiomarker approach to detect effects on the phototrophic compartment of biofilms indicating potential effects on the community structure.

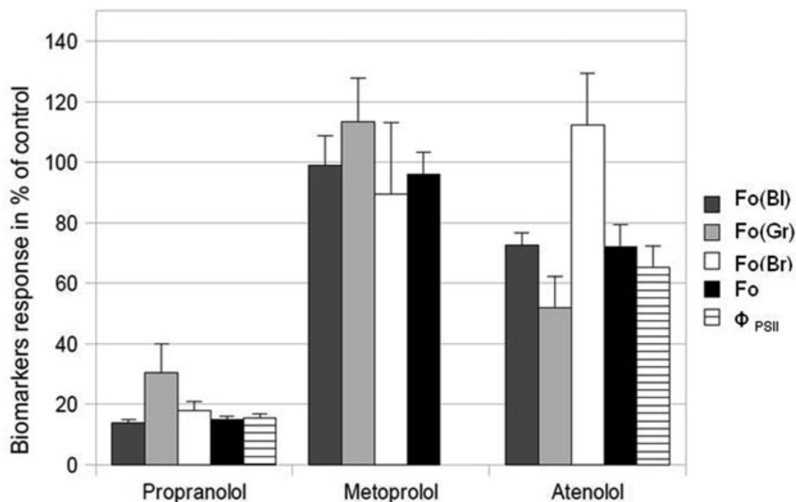

Fig. 5 Response of the minimal fluorescence yield of cyanobacteria [Fo(Bl), *dark gray*], green algae [Fo(Gr), *gray*], brown algae [Fo(Br), *white*] and the whole biofilm (Fo, *black*) and maximal quantum yield (Φ_{PSII}) of all biofilm (horizontal hatches), expressed in percentage of control, after 24 h of exposure to 531 µg/L of propranolol, 522 µg/L of metoprolol and 707,000 µg/L of atenolol. Error bars depict the standard error ($n = 3$). Modified from Bonnineau et al. [20]

Ricart et al. [23] studied the effects of the antimicrobial agent triclosan on fluvial communities. In this case, the PAM techniques were used to assess the effects of this antimicrobial agent on the biotic interaction between algae and bacteria within the biofilm community. The effective quantum yield (Φ'_{PSII}) was progressively reduced with increasing concentrations of toxicant up to 25% (Table 4). The NPQ parameter showed effects up to 70% (Table 4 and Fig. 6), indicating that the NPQ parameter was more sensitive to triclosan toxicity on biofilm than the Φ'_{PSII}. Triclosan effects on algae were also studied by Franz et al. [21] (Table 4). They used the Φ_{PSII} to compare differences in sensitivity to triclosan between algal monocultures and biofilm communities.

4.4 Field Studies

Pollution in fluvial ecosystems is related with the main activities that are carried out in their catchment. As a general rule, agricultural areas are more affected by organic chemicals (herbicides, insecticides, or fungicides), industrial and mining areas by metals, and urban areas by personal care products and pharmaceuticals via sewage treatment plant effluents [75, 77]. However, the predominant scenario found in most polluted rivers is a mixture of different chemicals. From an ecotoxicological point of view, mixture pollution implies that compounds with different chemical

Table 4 Summary of studies applying chl-*a* fluorescence techniques to assess the toxicity of toxicants with an unknown mode of action on fluvial biofilm communities

Toxicant	Biofilm community	Exposure (h)	Conc.	Φ_{PSII}	Φ'_{PSII}	NPQ	Fo	Φ'_{PSII} Fo (Bl)	Φ'_{PSII} Fo (Gr)	Φ'_{PSII} Fo (Br)	Reference
Atenolol	Experimental channels	24	707 mg/L	–	–			–	– –	n.s.	Bonnineau et al. [20]
Propranolol		24	531 µg/L	– – –	– – –			– –	– – –	– – –	
Metoprolol		24	522 µg/L		n.s.			n.s.	n.s.	n.s.	
Triclosan	Aquaria	24	270–17,300 µg/L	– – –							Franz et al. [21]
Triclosan	Experimental channels	48 h	0.05–500 µg/L		–	– – –					Ricart et al. [23]

The significant responses observed are indicated by "–" for inhibition or by "+" for increase and its approximate magnitude by "–" or "+" if it was slight (10–30% of control), by "– –" or "+ +" if it was moderate (30–50% of control) and by "– – –" or "+ + +" if it was high (>50% of control)
n.s. not significant (p>0.05)

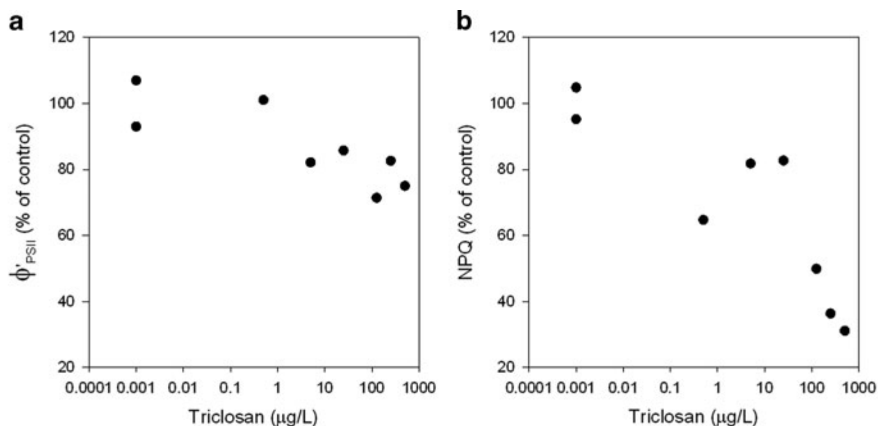

Fig. 6 Effects of triclosan on (**a**) effective quantum yield (Φ'_{PSII}) and (**b**) non-photochemical quenching (NPQ) of biofilms. Modified from Ricart et al. [23]

properties, MoA, and concentrations will coexist. Moreover, environmental factors such as light, nutrients, or flow regime may also influence the fate and effects of chemicals on fluvial communities. Due to its complexity, the effects of toxicants on biofilm communities have been addressed by different methodological approaches including periodic monitoring sampling, translocation experiments, or PICT approaches. In addition, different biofilm targeting endpoints have been used, including, in many cases, chl-*a* fluorescence parameters.

Ricart et al. [30] examined the presence of pesticides in the Llobregat river basin (Barcelona, Spain) and their effects on benthic communities (invertebrates and natural biofilms) (Table 5). Several biofilm metrics, including PAM parameters, were used as response variables to identify possible cause–effect relationships between pesticide pollution and biotic responses. Certain effects of organophosphates and phenylureas in both structural (chl-*a* content and Φ_{PSII}) and functional aspects (Φ'_{PSII}) of the biofilm community were suggested. The authors concluded that complemented with laboratory experiments, which are needed to confirm causality, this approach could be successfully incorporated into environmental risk assessments to better summarize biotic integrity and improve the ecological management. In the Morcille River, located in a vineyard area of France, several studies have been performed to evaluate pesticide effects on biofilms [78–80] (Table 5). Morin et al. [78] studied the structural changes in biofilm assemblages induced by the transfer of biofilm communities from two contaminated sites to a reference site, expecting a recovery of the translocated communities, either in structure or in diversity. The proportions of the different algal groups in biofilms were estimated by in vivo chl-*a* fluorescence measurements, observing that biofilm was mainly composed by diatoms before and after translocation (Table 5). In the same river Pesce et al. [79] used the Φ_{PSII} to evaluate individual and mixture effects of diuron and its main metabolites

Table 5 Review of field studies where chl-*a* fluorescence techniques have been applied to assess the effects of toxicants on photosynthesis performance of fluvial biofilm communities

Toxicant	Mode of action	River	Growth substrate	Experimental conditions	Φ_{PSII}	Φ'_{PSII}	NPQ	Fo	Fo (Bl)	Fo (Gr)	Fo (Br)	References
Cu	b	Ter River, Spain	Artificial substrata	Biofilm from different polluted sites (spring and summer) was transported to the laboratory for Cu and atrazine short-term toxicity tests		– – –						Navarro et al. [26]
Atrazine	a					– – –						
22 pesticides (mixture)	a and c	Llobregat River, Spain	Natural biofilm	Field monitoring of biofilm from different polluted sites during 2 years—natural exposure	–	–						Ricart et al. [30]
Atrazine	a	Seven streams from Catalonia Area, Spain	Artificial substrata	Biofilm from different polluted sites and during different seasons was transported to the laboratory for atrazine and Zn short-term toxicity tests		– – –						Guasch et al. [15]
Zinc	b					– –						
Atrazine	a	Ozanne River, France	Artificial substrata	Biofilm from different polluted sites and during different seasons was transported to the laboratory for atrazine and isoproturon toxicity tests—PICT concept	–							Dorigo and Leboulanger [65]
Isoproturon	a				–							
Zn, Cd, Ni, Fe (metal pollution)	b	Mort River, France	Natural biofilm	Biofilm from different polluted sites was evaluated—natural exposure		–						Bonnineau et al. [83]
Diuron, DCPMU and 3,4-DCA	a	Morcille River, France	Artificial substrata	Biofilm from different polluted sites, presenting a natural exposure, were sampled	–				– –			Pesce et al. [79, 80]
				Biofilm from different polluted sites was transported to the laboratory for short-term toxicity tests—PICT concept	– – –							

(continued)

Table 5 (continued)

Toxicant	Mode of action	River	Growth substrate	Experimental conditions	Φ_{PSII}	Φ'_{PSII}	NPQ	Fo	Fo (Bl)	Fo (Gr)	Fo (Br)	References
Pesticides	a and b	Morcille River, France	Artificial substrata	Biofilm from each sampling site was sampled before translocation to be structurally characterized—natural exposure					n.s.	n.s.	n.s.	Morin et al. [78]
Prometryn	a	Elbe River, Germany	Artificial substrata	Translocation experiment. Local and translocated biofilm was transported to the laboratory for short-term prometryn toxicity tests—PICT concept, structural characterization	− − −				+	+	− −	Rotter et al. [81]

The significant responses observed are indicated by "−" for inhibition or by "+" for increase and its approximate magnitude by "−" or "+" if it was slight (10–30% of control), by "− −" or "+ +" if it was moderate (30–50% of control) and by "− − −" or "+ + +" if it was high (>50% of control). The terminology used for mentioned the mode of action of each compound was: "a" for PSII inhibitors, "b" for photosynthesis inhibitors not directly targeting PSII and "c" for unknown mode of action on algae

n.s. not significant ($p > 0.05$)

(DCPMU and 3,4-DCA) on biofilms. They observed that diuron was the most toxic of the evaluated compounds and that biofilms from contaminated sites presented a higher tolerance to diuron and DCPMU than biofilms from a reference site. In another study, it was investigated how closely diuron tolerance acquisition by photoautrophic biofilm communities could reflect their previous in situ exposure to this herbicide. For this propose, the use of PICT (using the Φ_{PSII} as endpoint) together with multivariate statistical analyses was combined. A spatiotemporal variation in diuron tolerance capacities of photoautotrophic communities was observed, the biofilm from the most polluted site being the most tolerant [80] (Table 5). Similar tolerance results were found by Rotter et al. [81] in a study in the Elbe River where prometryn toxicity on biofilm was evaluated by combining a translocation experiment and the PICT approach (Table 5). They observed that biofilms from a polluted site presented a higher EC_{50} (based on Φ'_{PSII} measures) than biofilms from a reference site, suggesting a prometryn induction tolerance. The proportion of each group (cyanobacteria, green algae, and diatoms) in transferred and control biofilms was also measured by PAM fluorometry. In other studies [65, 82]) also used the PICT approach to investigate the toxicity of atrazine and isoproturon on biofilm from an herbicide polluted river (Ozanne River, France) (Table 5). Sampling was performed at different polluted sites and at different moments during the year. Using Φ_{PSII} as endpoint in short-term toxicity tests, differences in the EC_{50} between biofilms naturally exposed to different levels of atrazine and isoproturon could be observed.

Other field studies presenting organic and inorganic pollution were based on short-term toxicity tests to evaluate biofilms' sensitivity to toxic exposure [26] in the Ter River (NE, Spain) (Table 5). It was concluded that the short-term toxicity tests using the Φ'_{PSII} as a physiological endpoint may provide an early prospective quantification of the transient effects of a toxic compound on separate communities, predict which of these effects are not reversible, and determine their intensity. Guasch et al. [15] investigated the ecological and the structural parameters influencing Zn and atrazine toxicity on biofilm communities by testing the validity of the use of a short-term physiological method (based on the Φ'_{PSII} as microalgae endpoint) (Table 5). They concluded that short-term toxicity tests seem to be pertinent to assess atrazine toxicity on biofilms. In contrast, to assess Zn toxicity on biofilm the use of longer term toxicity tests was proposed to overcome the influence that biofilm thickness exerts on Zn diffusion and toxicity. Bonnineau et al. [83] assessed in situ the effects of metal pollution on Φ'_{PSII} in natural communities from the Riou-Mort river (France) by comparing reference and polluted sites. Slight effects were observed based on Φ'_{PSII} and marked effects were observed based on antioxidant enzymatic activities, supporting the low sensitivity of Φ'_{PSII} for the assessment in situ of the chronic effects of metals.

Based on the field studies reported here, it can be concluded that in field studies the responses of the chl-*a* fluorescence parameter could serve as an early warning signal of biological effects after acute exposure to photosystem II inhibitors, but in

regular monitoring PAM fluorometry may show low sensitivity or give false-negative signals [84] if the community is already adapted to the prevailing toxic exposure conditions. Tolerance induction evaluations and multibiomarker approaches, together with the use of appropriate multivariate statistical analyses, may partially overcome this limitation [85].

5 General Discussion and Perspectives

The main aim of this chapter was to analyze the pros and cons of the use of chl-*a* fluorescence techniques for water quality monitoring programs, based on biofilm communities, in the context of the WFD.

The use chl-*a* fluorescence parameters as biomarkers of toxicity on biofilm communities has increased in the last few years, illustrating their applicability to show early and long-term effects of toxicants at community level using functional and structural descriptors.

Differences in sensitivity are reported between chl-*a* fluorescence parameters. Focusing on functional parameters, the effective quantum yield (Φ'_{PSII}) seems to detect the effects on algae of toxic compounds targeting the PSII. Its use is well reported in both laboratory and field studies (Tables 2 and 5), supporting its use for these types of compounds. On the other hand, its sensitivity to chemicals with a mode of action different to the PSII is highly influenced by the time of exposure of the toxicant (short-term vs. long-term exposure) (Tables 3–5). In short-term studies, the Φ'_{PSII} seems to be a sensitive endpoint of toxicity but in long-term studies its sensitivity is often less obvious (Tables 3 and 5). This lack of sensitivity during long-term exposures could be related with a development of biofilm tolerance. Therefore, in long-term studies both in field and in laboratory conditions, supplementary methodological approaches, such as PICT tests based on Φ'_{PSII} may be used to show community adaptation.

The maximal photosynthetic capacity of biofilms (Φ_{PSII}) showed a similar sensitivity to chemicals as the Φ'_{PSII}. This parameter has been used more in ecotoxicological studies than the Φ'_{PSII}, probably due to its simplicity for being measured. The Φ_{PSII}, which is measured on dark-adapted samples, is independent of the light conditions prevailing during the incubation period. Therefore, it is more suitable than Φ'_{PSII} for comparing results of experiments using different light conditions [69]. The use of both photosynthetic parameters, Φ'_{PSII} and $\Phi_{PSII,}$ provides more information than only using one of these parameters, since both functional and structural alterations are evaluated.

The use of the NPQ parameter to assess chemical toxicity on biofilm is less documented than those reported above and applied only in microcosm studies. However, its use is promising due to its sensitivity to different types of toxicants, and also due to its complementarity to the more classical photosynthetic related parameters (Φ'_{PSII} and Φ_{PSII}) [23, 31, 69]. However, as the activation of the NPQ processes is restricted to a very specific period of time (from minutes to few hours

after the stress started), its use seems to be restricted to evaluate short-term toxicity. On the other hand, its sensitivity has also been observed in long-term studies. In these cases, it was linked to structural damage in the photosynthetic apparatus, specifically a NPQ reduction as a result of a modification of accessory pigments where NPQ processes occur [23, 31].

Finally, the use of the fast fluorescence techniques by PEA to assess chemical toxicity on biofilms is also recommended, indeed not yet applied. As this technique gives information about the kinetics of OJIP transients (Fig. 2), its application on biofilm could contribute to detect toxic effect of the PSII energy trapping or PSII antenna size and by this way complement the information obtained by PAM fluorometry. Its application on future ecotoxicological biofilm studies could be of special interest in studies focusing on the confirmation of the MoA of a determinate chemical on the photosynthetic processes of species composing biofilm community.

Concerning more structural chl-*a* fluorescence parameters, the minimal fluorescence parameter (Fo) has been applied for monitoring algal biomass (Tables 2–5) and biofilm growth rate [28, 31] in microcosms and field studies. It can be considered as a global indicator, which integrates effects of chemicals on different cellular metabolisms causing pigment damage and cell death. It has been successfully applied in ecotoxicological studies focusing on compounds that damage photosynthetic processes without targeting the PSII (Table 3). Since Fo is influenced by measuring conditions: gain, distance between sensor and the biofilm sample, or the measuring light intensity, absolute values are difficult to compare between different studies without using the same calibration. However, toxic effects on Fo, in ecotoxicological studies, are usually reported in comparison to a non-exposed community (in relative terms) to avoid this limitation. The measurements of Fo may present other limitations not related to the algal biomass or biofilm thickness. Fo may increase if the addition of a herbicide causes a transfer of the electron on PQ_B to the primary quinine acceptor (PQ_A) and displacement of PQ_B by the herbicide (Fig. 1); the reduced PQ_A leads to a higher Fo [86]. Consequently an overestimation of Fo could occur. Also, it is known that some chemicals emit fluorescence under excitation light (e.g., DCMU) that could not be distinguished from the fluorescence emitted from excited chl-*a* (Fo measure). In these cases, the use of Fo to assess algal biomass is not recommended. In field applications, this phenomenon could play an important role, since pollution is often due to a mixture of compounds of unknown origin. On the other hand, the greening effects could also contribute to limit the use of Fo for measuring algal biomass or algal growth. This phenomenon may occur due to an increase in the number of chl-*a* molecules per unit of cell, without increasing the number of cells [29, 87]. This situation could produce an increase of Fo values with no increase in the number of cells.

A significant portion of the ecotoxicological studies included in this chapter has incorporated the deconvolution of the chl-*a* fluorescence signal into the contribution of the main autotrophic groups (Tables 2–5). These measurements allow the main autotrophic groups of biofilms (structural approach) to be characterized.

Nowadays, PAM fluorometers which incorporate a four-wavelength excitation method to deconvolute these photosynthetic parameters of microalgal communities [Fo(Bl), Fo(Gr), and Fo(Br)] by incorporating spectral groups based on a single species pigment spectra. This calibration could be improved in the future if spectra used for calibrating the fluorometer are based on a mix of species from each group, based on the key species of each autotrophic group. This procedure could probably help to decrease uncertainty in these measurements.

6 Conclusions

Biofilms or periphyton communities are recognized for the Water Framework Directive (WFD, Directive 2000/60/EC) as a BQE. The use of chl-*a* fluorescence parameters as biofilm biomarkers allows to obtain a community approach which is much closer to the processes of an ecosystem than the use of single species tests. In fluvial ecosystems, the use of chl-*a* fluorescence parameters as a complementary set of biofilm biomarkers to the more traditional diatom indices applied so far is recommended due to its capacity to provide different warning signals of early and late toxicity effects. Its application is of special interest in the context of the WFD, where the development of new structural and functional parameters from the BQEs is required [88].

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