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Assessing and modeling nitrite inhibition in microalgae-bacteria consortia for wastewater treatment by means of photo-respirometric and chlorophyll fluorescence techniques

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ABSTRACT

Total nitrite (TNO_2 = HNO_2 + NO^-_2) accumulation due to the activity of ammonia-oxidizing bacteria (AOB) was monitored in microalgae-bacteria consortia, and the inhibitory effect of nitrite/free nitrous acid (NO_2-N/FNA) on microalgae photosynthesis and inhibition mechanism was studied. A culture of *Scenedesmus* was used to run two sets of batch reactors at different pH and TNO_2 concentrations to evaluate the toxic potential of NO_2-N and FNA. Photo-respirometric tests showed that NO_2-N accumulation has a negative impact on net oxygen production rate (OPR_{NET}). Chlorophyll a fluorescence analysis was used to examine the biochemical effects of NO_2-N stress and the mechanism of NO_2-N inhibition. The electron transport rate (ETR), non-photochemical quenching (NPQ), and JIP-test revealed that the electron transport chain between Photosystems II and I (PS II and PS I) was hindered at NO_2-N concentrations above 25 g N m^{-3}. Electron acceptor Q_A was not able to reoxidize and could not transfer electrons to the next electron acceptor, Q_B, accumulating P^+_{680} (excited PS II reaction center) and limiting oxygen production.

A semi-continuous reactor containing a *Scenedesmus* culture was monitored by photo-respirometry tests and Chlorophyll a fluorescence to calibrate NO_2-N inhibition (5 – 35 g N m^{-3}). Non-competitive inhibition and Hill-type models were compared to select the best-fitting inhibition equations. Inhibition was correctly modeled by the Hill-type model and a half inhibition constant (K_I) for OPR_{NET}, NPQ, maximum photosynthetic rate (ETR_{MAX}) and the performance index PI_{ABS} was 23.7 ± 1.2, 26.36 ± 1.10, 39 ± 2 and 26.5 ± 0.4, respectively.

Keywords: Chlorophyll a fluorescence; Hill-type model; Microalgae; Nitrite inhibition; Photo-respirometry; Wastewater treatment.

HIGHLIGHTS

- Nitrite and free nitrous acid inhibitory effects of photosynthesis were investigated by photo-respirometric tests.
- Nitrite, rather than free nitrous acid, has a negative impact on photosynthesis.
- Nitrite stress suppresses the electron transport on the donor and acceptor side of the Photosystem II reaction center.
- Nitrite inhibition was successfully modelled by Hill-type equation and the inhibition constant was calculated with different photosynthetic indexes.

NOMENCLATURE

Chl a	Chlorophyll a
PAR	Photosynthetically active radiation (µmol m^{-2} s^{-1})
PS II	Photosystems II
PS I	Photosystems I
LHC II	Light-harvesting complex of PS II
LHC I	Light-harvesting complex of PS I
P_{680}	PS II reaction center
PQ	Plastoquinone
Q_A	PSII primary quinone acceptor
Q_B	PSII secondary quinone acceptor
cytb6f	Complex cytochrome b6f
OEC	Oxygen-evolving complex
RC	Reaction Center
TNO_2	Total nitrite ($TNO_2 = HNO_2 + NO^-_2$) (g N m^{-3})
FNA	Free nitrous acid (g N m^{-3})
K_I	Half inhibition constant (g N m^{-3})
n	Hill coefficient
OPR_{NET}	Net oxygen production rate (mg O_2 g VSS^{-1} h^{-1})
ETR	Electron transport rate
ETR_{MAX}	Maximum photosynthetic rate (µmol m^{-2} s^{-1})

α	Initial slope of ETR curve (electrons/photon)
E_K	Saturating irradiance of photosynthesis (μmol m^{-2} s^{-1}).
NPQ	Non-photochemical quenching
F_o	Minimum fluorescence level in dark-adapted state (Relative units)
F_m	Maximum fluorescence in the dark-adapted state (Relative units)
F_v	Variable fluorescence in the dark-adapted state (Relative units)
F_J	Fluorescence at the J-peak of OJIP curve (Relative units)
F_tL	Instantaneous fluorescence in the light adapted phase (Relative units)
F_ML	Effective quantum yield of photosynthesis in the light-adapted state (Relative units)
Area	Total complementary area between fluorescence induction curve and F = F_m
V_J	Relative variable fluorescence at the J-peak of OJIP curve
F_V/F_M	Maximum quantum yield of PS II photochemistry
M_o	Approximated initial slope (ms^{-1}) of the fluorescence transient V = f(*t*)
S_M	Normalized total complementary area above the OJIP transient
ABS/RC	Absorption flux per RC
TR_o/RC	Trapped energy flux per RC
DI_o/RC	Dissipated energy flux per RC
φ_{Po}	Maximum quantum yield of primary photochemistry (at *t* = 0)
φ_{Eo}	Quantum yield of electron transport (at *t* = 0)
ψ_{Eo}	Probability (at *t* = 0) that a trapped exciton moves an electron into the electron transport chain beyong Q_A^-
φ_{Do}	Quantum yield (at *t* = 0) of energy dissipation
δ_{Ro}	Efficiency/Probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PS I acceptor side
φ_{Ro}	Quantuym yield for reduction of end electron acceptors at the PS I acceptor side
PI_{ABS}	Performance index; (potential) for energy conservation from photons absorbed by PSII to reduce Q_A
PI_{TOT}	Performance index; (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors

1. INTRODUCTION

Microalgae-bacteria based wastewater treatment systems have emerged as a sustainable and feasible alternative to remove the main pollutants from wastewater, specifically organic matter and nutrients (Bankston et al., 2020; Fito and Alemu, 2018; Mujtaba and Lee, 2017; Robles et al., 2020; Wang et al., 2017). Microalgae-bacteria consortia are supported mainly by a mutualistic interaction between

microalgae and heterotrophic bacteria. The oxygen produced by photosynthesis is used to oxidize organic matter by heterotrophic bacteria, while the carbon dioxide released by organic matter oxidation is used as a carbon source for microalgae (Fito and Alemu, 2018). However, other bacterial populations e.g. ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing (NOB) (nitrifying bacteria), develop in this biological community. The combined processes of ammonium (NH_4-N) assimilation by microalgae and nitrification can improve nitrogen removal rate in wastewater (Akizuki et al., 2019; Rada-Ariza et al., 2017), while negative interactions can also develop between AOB and microalgae communities. Although competition for NH_4-N is the most frequently studied negative interaction (Galès et al., 2019; González-Camejo et al., 2019; Risgaard-Petersen et al., 2004), amensalistic interactions also develop, e.g., microalgae inhibition by the final product of AOB metabolism, nitrite (NO_2-N) (González-Camejo et al., 2020). Admiraal (1977) detected a decrease in the photosynthetic rate of diatoms when incubated in media with NO_2-N concentrations between 10 and 50 g N m^{-3}. González-Camejo et al. (2020) reported that an NO_2-N concentration ranging from 5 to 20 g N m^{-3} inhibits microalgae metabolism in terms of biomass productivity and nutrient removal. Although NO_2-N can be oxidized by NOB, Akizuki et al. (2019), González-Camejo et al. (2020b, 2017) and Van Den Hende et al. (2016) reported that NO_2-N can accumulate and reach concentrations of 15 to 50 g N m^{-3} in microalgae-bacteria consortia.

NO_2-N ion is related to nitrous acid (HNO_2) through acid-base equilibrium and, therefore, the relationship between NO_2-N and free HNO_2 (FNA) is highly dependent on pH ($NO_2^- + H_2O \leftrightarrow HNO_2 + OH^-$; pK_a = 3.16 (Da Silva et al., 2006), for example, the concentration of HNO_2 at pH 4.5 and 8 (25ºC) accounts for 4.2% and 0.001%, respectively). Although microalgae-bacteria cultures do not provide the necessary conditions for obtaining a high FNA concentration, the protonated species has been reported to inhibit indigenous wastewater microorganisms at very low concentrations (Blackburne et al., 2007; Claros et al., 2013; Pijuan et al., 2010; Yang et al., 2003), so that FNA should be discarded as the true inhibitor of microalgae metabolism instead of NO_2-N.

The TNO_2 ($TNO_2 = HNO_2 + NO^-_2$) inhibition of photosynthesis can be assessed in the following ways: by batch growth experiments, measuring nutrient removal rate or by coupling photo-respirometry tests with *Chlorophyll a* (*Chl a*) fluorescence measures (Perales-Vela et al., 2007). Photo-respirometry tests

can be successfully applied to identify potential inhibitory effects through the oxygen production rate (Rossi et al., 2020b) but they do not provide information on the inhibition mechanism in microalgal metabolism. *Chl a* concentration provides plenty of information about the Photosystem II (PS II) performance and electron transport chain (Strasser et al., 2004). In microalgae cultures, *Chl a* fluorescence measurement has been proposed as a reliable tool to study changes in primary photosynthetic processes (light-dependent reactions) due to the inhibitory compounds: free ammonia, (which negatively impacts PS II and Photosystem I (PS I), electron transport chain, the oxygen-evolving complex and dark respiration (Li et al., 2019; Markou et al., 2016)), atrazine, (damaging PS II reaction center, suppressing the electron transport chain and acting on absorption, transfer and utilization of light (Sun et al., 2020)), copper oxide nanoparticles, (which damages the oxygen-evolving complex and inhibits the electron transport chain (Che et al., 2018)), polystyrene microplastics, (which damages PS II (Li et al., 2020)), volatile organic compounds, (reducing concentration of PS II reaction centers, suppressing the electron transport chain and acting on light absorption (Zhao et al., 2016)) and herbicides, (which damages PS II (Magnusson et al., 2008)). The combination of these techniques can thus provide a rapid and simple assessment of TNO_2 inhibition of photosynthetic processes.

Mathematical models have been widely used to study the simultaneous effects of different environmental and operational variables on the activity of microalgae-bacteria consortia (Sánchez-Zurano et al., 2021; Sánchez-Zurano et al., 2021a; Solimeno et al., 2017). However, the biokinetics of bacteria and eukaryotic organisms are developed independently and some negative interactions between the communities are not included. Inhibition processes should be included, as for example microalgae TNO_2 inhibition reported by González-Camejo et al. (2020), which must first be modeled and calibrated.

The aims of this study were: (I) to confirm that NO_2-N inhibits microalgae metabolism; (II) to analyze the inhibition mechanism of NO_2-N on photosynthesis; and (III) to propose a mathematical model to describe toxic species inhibition. To the best of the authors' knowledge, this is the first study to analyze the effect of TNO_2 on PS II and the electron transport chain activity of microalgae-bacteria consortia from an outdoor raceway pond reactor fed with real wastewater.

2. MATERIALS AND METHODS

2.1. Microorganism and wastewater

Microalgae-bacteria consortia were obtained from an outdoor pilot-scale membrane high rate algal pond (MHRAP) in the "Cuenca del Carraixet" Wastewater Treatment Plant (WWTP), Valencia (Spain). This plant mainly consisted of one HRAP connected to a membrane tank (MT). The HRAP had a surface area of 1.275 m^2 (2.55 x 0.5 m) and an operating depth of 0.25 m. A single six-blade paddle wheel was used to obtain the complete mixing of the culture medium. The MHRAP was fed with effluent from the pre-treatment of the above mentioned WWTP, having demonstrated good characteristics as the growth substrate for microalgae-bacteria consortia. The average characteristics of the wastewater were 34 ± 5 g N m^{-3} of nitrogen (mainly ammonium; i.e.>98% of total soluble nitrogen), 4.20 ± 1.04 g P m^{-3} of phosphorus, 297 ± 38 g TSS m^{-3} of total suspended solids (TSS), 360 ± g COD m^{-3} of soluble chemical oxygen demand (sCOD) and the alkalinity was 340 ± 49 mg $CaCO_3$ L^{-1}.

Microalgae-bacteria consortia were observed under Leica DM2500 microscope. The term "microalgae" will be used to describe eukaryotic microalgae, and therefore cyanobacteria are not included. The dominant indigenous microalgae morphology was associated with different *Scenedesmus* genera. Microalgal community was composed of >99% different *Scenedesmus* morphologies and <1% spherical unicellular microalgae cells. Mixed culture obtained by González-Camejo et al. (2020a) was composed of *Scenedesmus* and *Chlorella* using the same matrix culture, so that observed unicellular microalgae cells could be identified as *Chlorella*. No changes in microalgal community composition were observed during the study. Cyanobacteria were not observed. Microalgae were not observed forming flocs but free in the culture broth (Supplementary Data). Bacteria never accounted for more than 2% of the total biomass, resulting in a microalgae-bacteria ratio around 0.98.

2.2. Experimental set up

Two different sets of experiments were performed: (I) lab-scale tests to confirm the species of TNO_2 that inhibits microalgal metabolism and its mechanism of inhibition; and (II) lab-scale tests to calibrate the parameters of the model related to the inhibition process.

2.2.1. Inhibition tests experimental setup

Batch tests were carried out to confirm that NO_2-N inhibits photosynthesis, but mainly to analyze its inhibition mechanism: the net oxygen production rate (OPR_{NET}), electron transport rate (ETR) curve, non-photochemical quenching (NPQ) and the fluorescence transient kinetics. Three sets of three batch tests were performed with three different TNO_2 concentrations and two pH values. All the experiments were performed in triplicate. Three TNO_2 concentrations and one pH value were studied in each test (0, 25 and 50 g N m^{-3} with pH set at 4.5 or 8 (Table 1)). The following nomenclature was used to refer and identify each batch test: B(working pH)_(TNO_2 concentration), so that a batch reactor operated at a pH value of 8 and a TNO_2 concentration of 25 g N m^{-3} was referred to as B8_25. Values of pH 4.5 and 8 were selected according to FNA concentration. The NO_2-N effect on photosynthesis can be analyzed at pH 8 since the chemical equilibrium is completely shifted towards the NO_2-N species and FNA concentration is negligible. FNA's effects on microalgal photosynthesis were studied in the batch experiments operated at pH 4.5.

In each set, 3L of microalgae-bacteria consortia culture from the MHRAP pilot plant was centrifuged at 5000xg (Eppendorf AG 22331, Hamburg) and resuspended with synthetic wastewater with the following composition (adapted medium from (Test No. 201: Alga, Growth Inhibition Test, 2006): NH_4Cl, 133.72 g m^{-3}; KH_2PO_4, 17.66 g m^{-3}; $MgSO_4 \cdot 7H_2O$, 28.99 g m^{-3}; $CaCl_2 \cdot 2H_20$, 37.67 g m^{-3}; $FeCl_3 \cdot 6H_2O$, 17.16 g m^{-3}; H_3BO_3, 1.72 g m^{-3}; $CuSO_4 \cdot 5H_2O$, 0.15 g m^{-3}; KI, 0.25 g m^{-3}; $MnCl_2 \cdot 4H_2O$, 1.02 g m^{-3}; $Na_2MoO_4 \cdot 2H_2O$, 1.07 g m^{-3}; $ZnSO_4 \cdot 7H_2O$, 4.21 g m^{-3} and $CoCl_2 \cdot 6H_2O$ 0.66 g m^{-3} and an alkalinity of 297 ± 37 mg $CaCO_3$ L^{-1}. Allylthiourea (ATU) at 10 g m^{-3} and $KClO_3$ at 1.2 g L^{-1} (Rossi et al., 2018) were also added to the synthetic wastewater to inhibit ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) growth, respectively, and to keep TNO_2 concentration constant during the tests. Hydroxylamine is widely used to inhibit NOB (Zhao et al., 2021), but unlike ATU and $KClO_3$, it also inhibits photosynthetic activity (Supplementary Data). The biomass was resuspended to set optical density at 680 nm (OD_{680}) close to 0.5, providing comparable light attenuation. 1.5 L of the resuspended culture was distributed between three batch reactors (500 mL working volume) operated in parallel. A nitrite standard solution (sodium nitrite) of 1000 g NO_2 m^{-3} was added to the batch reactors at the beginning of the experiments to achieve an initial TNO_2 concentration of 0, 25 and 50 g N m^{-3}. As NO_2-N and FNA concentrations are highly dependent on pH,

concentrations of both species were obtained (Table 1) according to culture temperature and pH using Visual MINTEQ 3.1 software.

The batch reactors were equipped with: a dissolved oxygen probe (WTW CellOx 330i) connected to an oximeter (Oxi 320, WTW, Germany) recording both dissolved oxygen (DO) and temperature data every 5 s, a lighting system made of 2 cool-white LED lamps (Seven ON LED 2 x 11W) to supply a light photosynthetically active radiation (PAR) of 100 µmol m^{-2} s^{-1}, an on-off electro-valve supplied air to keep the O_2 concentration in the 80 to 120% saturation range, and a magnetic stirrer system ran at 150 rpm to ensure homogeneous conditions. The reactor was placed in a thermostatic chamber keeping the culture temperature at 26.1 ± 0.2 ºC (which is in the optimal range for microalgae growth 25-30 ºC (González-Camejo et al., 2019; Rossi et al., 2020a)).

The pH was controlled by 0.5 M HCl or 0.2 M NaOH (Pijuan et al., 2010) addition using a fuzzy-logic controller and monitored by a pH probe connected to a multiparametric analyzer (CONSORT C832, Belgium). The volume added was recorded to monitor the dilution effect.

The sets of experiments were performed in triplicate and included a control reactor, i.e. the nitrite standard solution was not added but the reactor was operated under the same culture conditions as the other batch reactors. Photosynthetic activity was analyzed by comparing the reactors at different TNO_2 levels with the control reactor of each set. This experimental procedure reduces the variability of microalgae biomass on different experimental days and the effect of pH on the biological parameters analyzed.

Table 1. Experimental conditions applied in each experimental test.

	Set 1			Set 2		
Batch reactor	B4.5_0 (control)	B4.5_25	B4.5_50	B8_0 (control)	B8_25	B8_50
pH	4.3 ± 0.4	4.4 ± 0.2	4.5 ± 0.3	8.0 ± 0.3	7.9 ± 0.3	8.2 ± 0.2
NO_2-N (g N m^{-3})	0	23.96 ± 0.14	47.95 ± 0.09	0	25.3 ± 0.7	49.7 ± 0.5
HNO_2 (g N m^{-3})	0	1.04 ± 0.11	2.04 ± 0.04	0	0.00	0.00

2.2.1.1. Net oxygen production rate (OPR_{NET})

The 3 batch reactors described in Section 2.2.1 were operated for 5 days, in triplicate. The OPR_{NET} was obtained by performing photo-respirometric tests, using a protocol adapted from Rossi et al. (2018) and Sánchez-Zurano et al. (2020) (Fig. 1A).

1. A 500 mL aliquot of resuspended microalgae culture (at OD_{680} close to 0.5) was inoculated into each reactor.
2. Nitrite standard solution was added to achieve a TNO_2 concentration of 25 and 50 g N m^{-3}, taking the dilution effect into account.
3. The batch reactors were subjected to 20-min light and dark periods (in 10-min phases) throughout the 5 experimental days. DO was measured and registered every 10 s. As recommended by Sánchez-Zurano et al. (2020a), the first minute of light and dark exposure was disgregaded as was considered the adaptation time. The electro-valve was open and air was provided between the light and dark phases to keep O_2 concentration in the 80 to 120 %Sat range.

DO generation was expected in the light phases as a result of microalgae photosynthesis, while the oxygen was consumed by respiration in the dark phase. Nitrifiying bacteria activity was neglected due to the addition of AOB and NOB inhibitors (ATU and $KClO_3$, respectively), as was the aerobic activity of heterotrophic bacteria, since the used synthetic wastewater used did not contain organic matter and the biomass culture sCOD was less than 15 g COD m^{-3}. The OPR_{NET} (mg O_2 g VSS^{-1} h^{-1}) was calculated as the sum between the monitored values (i.e., specifying whether the slope is positive or negative) of the oxygen production rate (OPR) slope in the light phase and the oxygen uptake rate (OUR) slope in the dark phase, divided by the dry wieght of total biomass in the sample (Eq. 1) (i.e. volatile suspended solids (VSS), which were measured according to the Standard Methods). The OUR is the oxygen consumed by the respiration of microalgae and endogenous respiration of bacteria. The MHRAP pilot plant was monitored and controlled during all experimental periods. VSS remained stable during the whole period at an average concentration of 325 ± 45 g VSS m-3. The biological culture was periodically examined under the microscope. Bacteria never accounted for more than 2% of the total biomass, thus, VSS were considered to represent microalgae biomass. Similary, Luo et al. (2018) reported that bactertia accounted for only 0.2-3.5% of microalgae-bacteria consortium biomass. For this reason, the whole biomass measured as VSS was considered to be composed solely of microalgae. The oxygen mass transfer coefficient (K_La) was calculated to correct the influence of oxygen desorption on the photo-respirometric measures (Eq. 2 and Eq. 3). The K_La coefficient was evaluated by performing an abiotic test in distilled water (in triplicate) in the same chemical-physical

conditions set during the experiments. Destilled water was placed in the batch reactor and oxygen concentration was increased to 130%Sat by bubbling air. Then, aeration was stopped and oxygen concenrtation was recorded during 3 hours. The minimum residual sum of squared errors was used to match dynamic mass balance for DO with Eq. 2 (González-Camejo et al., 2020) and K_La value of 0.27 ± 0.04 h^{-1} was obtained at 25ºC.

$$\mathrm{OPR_{NET}} = \frac{\mathrm{OPR} + \mathrm{OUR}}{\mathrm{VSS}} \quad \text{Eq.1}$$

$$\frac{\mathrm{d(DO)}}{\mathrm{dt}} = \mathrm{K_La} \cdot (\mathrm{DO_{SAT}} - \mathrm{DO}) \quad \text{Eq.2}$$

$$\mathrm{DO_{SAT}} = \mathrm{pO_2} \cdot \mathrm{K_{H,O_2}(T)} = \mathrm{pO_2} \cdot \mathrm{K_{H,O_2,REF}} \cdot \exp\left(-\frac{-\Delta_{\mathrm{SOL}}\mathrm{H}}{\mathrm{R}} \cdot \left(\frac{1}{\mathrm{T}} - \frac{1}{\mathrm{T_{REF}}}\right)\right) \quad \text{Eq.3}$$

where OPR_{NET} is the net oxygen production rate [g O_2 g VSS^{-1} h^{-1}], *OPR* is the oxygen production rate, value [g O_2 m^{-3} h^{-1}], *OUR* is the oxygen uptake rate, value [g O_2 m^{-3} h^{-1}], *VSS* is the volatile suspended solids [g VSS m^{-3}], K_La is the oxygen mass transfer coefficient (h^{-1}), DO_{SAT} is the DO saturation concentration (g O_2 m^{-3}) at tempertature *T* (K) and the partial pressure of oxygen in atmosphere pO_2 (Atm), and $K_{H,O2}$(T) = 40.5 [mg O_2 L^{-1} Atm^{-3}] is the Henry's law solubility constant for DO at temperature T (Sander, 2015) and $\Delta_{\mathrm{SOL}}\mathrm{H/R}$ = 1200 [K] (tabulated) is the enthalpy of dissolution divided for the univeral gas constant (Sander, 2015). The reference temperature (T_{REF}), and pO_2 were 298.15 K and 0.21 atm.

The OPR_{NET} was used to confirm that NO_2-N inhibits microalgal photosynthesis, while *Chl a* fluoresecence was used to analyze the mechanism of inhibitor action.

2.2.1.2. Electron transport rate (ETR) curve

The ETR curve was recorded in dark-adapted samples (20 min) using the Light curve test (LC1) protocol of the fluorometer AquaPen-C AP-C 100 (Photon Systems Instruments, Czech Republic). This protocol is based on successive measurements of the microalgae sample exposed to a stepwise increase in actinic light intensity (red-light diode). The effective quantum yields of PS II are determined under 6 actinic light intensities: 10, 20, 50,100, 300 and 500 μmol·m^{-2}·s^{-1}, with 60 s phase duration (PSI (Photon Systems Instruments), 2017). The ETR curve was calculated using the quantum yields of photosynthesis derived from the LC1 protocol and the number of absorbed photons per *Chl a* and time according to Eq. 4.

$$ETR = \Phi_{PS\,II} \cdot E \cdot 0.5 \quad \text{Eq. 4}$$

where $\Phi_{PS\,II}$ is the quantum yield of PS II, *E* is the irradiance of the actinic light [μmol·m^{-2}·s^{-1}] and *0.5* is the fraction of absorbed quanta direct to PS II, i.e. the radiant energy is absorbed by PS I and PS II equally (Yamori et al., 2011).

The nonlinear model described by Serôdio et al. (2013) was applied to match the ETR curves obtained and to estimate the following key parameters: (I) maximum photosynthetic rate (ETR_{MAX}), the maximum electron transport rate through the PS II (μmol m^{-2} s^{-1}); (II) the initial slope of ETR curve (α), the quantum efficiency of photosynthetic electron transport (electrons/photon); and (III): the saturating irradiance of photosynthesis (E_K) (μmol m^{-2} s^{-1}).

After 5 experimental days, an aliquot of 3 mL from each batch reactor was kept in darkness for 20 min before applying the LC1 protocol.

2.2.1.3. Non-photochemical quenching (NPQ)

NPQ was measured in dark-adapted samples (20 min) following the AquaPen-C AP-C 100 NPQ2 protocol. The NPQ protocol is divided in two parts, one measuring fluorescence under dark-adapted and a second under light-adapted conditions. The protocol begins by giving a measuring light to obtain the minimum fluorescence level in a dark-adapted state (F_o). A short saturating light flash is then applied to reduce the plastoquinone (PQ) pool and the maximum fluorescence is measured in the dark-adapted state (F_m). After a short-term relaxation in the dark, the microalgae sample is exposed to light actinic for hundreds of seconds to elicit a transient of the Kautsky effect measuring the instantaneous Chl fluorescence in the light-adapted phase (F_tL) (Stirbet and Govindjee, 2011). An additional saturating flash sequence is applied on top of the actinic light to measure NPQ and the effective quantum yield of photosynthesis in the light-adapted state (F_ML). After exposure to continuous illumination, NPQ relaxation is determined by saturating pulses applied in the dark, measuring maximum Chl fluorescence and instantaneous Chl fluorescence in the dark adapted phase (F_tD and F_MD, respectively) (PSI (Photon Systems Instruments), 2017).

NPQ was measured dark-adapted samples (20 min) from the batch reactors after 5 experimental days.

248 2.2.1.4. Fluorescence transient (JIP-test)

249 Transient chlorophyll fluorescence induction in dark-adapted samples was measured by the AquaPen-C
250 AP-C 100 JIP-test. The fast fluorescence induction curve was recorded in 50 µs - 2 s time range with a
251 saturating light of 3000 µmol·m^{-2}·s^{-1} of red-orange light (620 nm). The fluorescence transient starts at the
252 phase O (at 50 µs), passes through two intermediate, phases J and I, (at 2 and 60 ms, respectively) and
253 reaches the maximum fluorescence in the phase P. The set of phases is called the OJIP curve.

254 To better evaluate the events reflected in the OJIP curve and between the O-J, J-I and I-P phases, the
255 fluorescence data were normalized (between the minimum and maximum fluorescence intensity, F_o and F_m,
256 respecitvely) and presented as the relative variable fluorescence kinetics at any time (Eq. 5) and as a kinetic
257 profile of the difference between the control reactor and the other two batch reactors in the same set (Eq. 6)
258 (Gomes et al., 2012).

$$V_{OP} = \frac{(F_t - F_o)}{(F_m - F_o)} \quad \text{Eq. 5}$$

$$\Delta V_{OP} = (V_{OP} - V_{OP-\text{control reactor}}) \quad \text{Eq. 6}$$

259 where F_t is the instantaneous chlorophyll fluorescence at any time t (relative units); F_o is the minimum
260 chlorophyll fluorescence (relative units); F_m is the maximum chlorophyll fluorescence (relative units); and
261 V_{OP} is the relative variable fluorescence (relative units).

262 The JIP-test includes the determination of the photochemical parameters listed in Table 2.

263 JIP-test was recorded using a 3 mL dark-adapted sample (20 min) from the batch reactors after the 5
264 experimental days.

265 Table 2. Formula and definition of JIP-test parameters.

Data extracted from the recorded fluorescence transient OJIP	
F_o	Minimal reliable recorded fluorescence at 50 µs (Relative units)
F_J	Fluorescence at the J-peak of OJIP curve (Relative units)
F_t	Fluorescence at time *t* after actinic illumination onset (Relative units)
F_m	Maximal recorded fluorescene at the peak P of OJIP curve (Relative units)
Area	Total complementary area between fluorescence induction curve and F = F_m
Fluorescence parameters derived from the extracted data	
$V_J = \frac{F_J - F_o}{F_m - F_o}$	Relative variable fluorescence at the J-peak of OJIP curve

$\frac{F_V}{F_m} = \frac{F_m - F_o}{F_m}$	Maximum quantum yield of PS II photochemistry
$M_o = \frac{4(F_{300} - F_o)}{F_m - F_o}$	Approximated initial slope (ms^{-1}) of the fluorescence transient V = f(*t*)
$S_M = \frac{Area}{F_m - F_o}$	Normalized total complementary area above the OJIP transient
Specific energy fluxes, per Q_A-reducing PS II reaction center (RC)	
$\frac{ABS}{RC} = M_o \cdot \left(\frac{1}{V_J}\right) \cdot \left(\frac{1}{\varphi_{Po}}\right)$	Absorption flux per RC
$\frac{TR_o}{RC} = M_o \cdot \left(\frac{1}{V_J}\right)$	Trapped energy flux per RC
$\frac{DI_o}{RC} = \left(\frac{ABS}{RC}\right) - \left(\frac{TR_o}{RC}\right)$	Dissipated energy flux per RC
Yields or flux ratio	
$\varphi_{Po} = 1 - \frac{F_o}{F_M}$	Maximum quantum yield of primary photochemistry (at *t* = 0)
$\varphi_{Eo} = \left(1 - \frac{F_o}{F_M}\right) \cdot (1 - V_J)$	Quantum yield of electron transport (at *t* = 0)
$\psi_{Eo} = 1 - V_J$	Probability (at *t* = 0) that a trapped exciton moves an electron into the electron transport chain beyong Q_A^-
$\varphi_{Do} = \left(\frac{F_o}{F_M}\right)$	Quantum yield (at *t* = 0) of energy dissipation
$\delta_{Ro} = \frac{1 - V_I}{1 - V_J}$	Efficiency/Probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PS I acceptor side
$\varphi_{Ro} = \left(1 - \frac{F_o}{F_M}\right) \cdot (1 - V_I)$	Quantuym yield for reduction of end electron acceptors at the PS I acceptor side
Performance indexes (PI) at *t* = 0	
$PI_{ABS} = \frac{RC}{ABS} \cdot \frac{\varphi_{Po}}{1 - \varphi_{Po}} \cdot \frac{\psi_{Eo}}{1 - \psi_{Eo}}$	Performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors
$PI_{TOTAL} = \frac{PI_{ABS} \cdot \varphi_{Ro}}{(1 - \varphi_{Ro})}$	Performance index (potential) for energy conservation from exciton to the reduction of PS I end acceptors

2.2.2. Model calibration setup

2.2.2.1. Semi-continuous reactor

A lab-scale reactor was operated in semi-continuous mode to promote controlled conditions (temperature, light intensity, pH, and nutrients and CO_2 availability) and to calibrate the parameters of the mathematical model related to photosynthesis inhibition by TNO_2 and achieve the optimal non-stressed biomass for calibration (i.e. microalgae activity was not limited by environmental conditions). The lab-scale reactor consisted of an 8-L cylindrical methacrylate tank (20-cm internal diameter) and was filled with 33% of substrate (synthetic wastewater) and 67% of microalgae-bacteria consortia from MHRAP pilot plant (see

275 section 2.1). Air-stirred at 0.7 vvm through four fine bubble diffusers on the bottom to homogenize the culture
276 and avoid biofilm on the reactor walls and biomass sedimentation. Pure CO_2 (99.9%) was injected from a
277 pressurized cylinder at 1.5-2.0 bar pressure into the air flow to keep reactor pH constant at 7.5 ± 0.4. An
278 on-off electro-valve synchronized with the pH measurements recorded by the data acquisition system (a
279 temperature-pH probe connected to a multiparametric analyzer, CONSORT C832, Belgium) was opened
280 for 2 s when the pH exceeded the set point value of 7.5. The reactor was placed in a thermostatic chamber
281 keeping the culture temperature at 25.3 ± 0.8 ºC. Five LED lamps (T8 LED-Tube 9 W) were placed around
282 the reactor to provide a continuous light PAR of 154 ± 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the reactor surface. The reactor
283 was sealed but not hermetic, to avoid extreme overpressure and oxygen concentration was kept close to
284 saturation levels (111 ± 7 %Sat). DO was measured by a Cellox 330i electrode (WTW, Germany) connected
285 to an oximeter (Oxi 320, SET WTW, Germany).
286 The reactor was operated in semi-continuous mode, maintaining 4 days of hydraulic retention time (HRT).
287 Every 3 hours, 0.250 L of biomass culture was taken from the reactor and replaced with synthetic wastewater
288 (Section 2.2.1).

289 2.2.2.2. Calibration reactor

290 The concentration levels of the tested TNO_2 was calibrated by the semi-continuous reactor biomass culture.
291 The calibration procedure was carried out in a 0.5 L batch reactor, inside the same thermostatic chamber
292 as the semi-continuous reactor, providing a culture temperature of 25.7 ± 1.2 ºC. An on-off valve was used
293 to add pure CO_2 (99.9%) for 1 s when the pH exceeded the set point value of 7.5, keeping constant pH at
294 7.5 ± 0.6. The microalgae culture was stirred at 250 rpm to ensure homogenization and avoid biomass
295 sedimentation. K_La was 0.19 h^{-1} (the protocol applied is described in Section 2.2.1.1.). Two LED lamps
296 (Seven ON LED 11 W) were placed over the reactor to provide an average light PAR of 162 ± 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$
297 on the reactor surface. The batch reactor was equipped with a Cellox 330i electrode (WTW, Germany)
298 connected to an oximeter (Oxi 320, SET WTW, Germany) connected to a personal computer.

299 Ten different experiments were performed in triplicate at different TNO_2 concentrations ranging from 2.5 to
300 35 g N m^{-3} (calibration TNO_2 concentrations studied were 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30 and 35 g N m^{-3}).
301 An aliquot of 0.5 L of microalgae sample was placed inside the batch reactor and nitrite standard solution

302 was added to achieve TNO_2 levels. Concentration of NO_2 and FNA were calculated on Visual MINTEQ 3.1
303 software. After 20 min of adaptation to the TNO_2 concentration, the microalgae culture was exposed to 5-
304 min light-dark cycles (both lasted 2.5 minutes and 4 cycles were performed) to record DO concentration
305 every 10 s performed between 80 and 120 %Sat. The first minute of light and dark exposure was
306 disgregaded as it was considered the adaptation time (Sánchez-Zurano et al., 2020). Microalgae OPR_{NET},
307 ETR curve, NPQ, and the JIP-test were measured as described in Section 2.2.1 after 20 min of NO_2/FNA
308 addition.
309 The 10 inhibition assay sets were performed in triplicate. Since the culture volume of the semi-continuous
310 reactor was limited to 8 L, the assays were carried out on different days. The stability of the semi-continous
311 reactor was monitorized according to VSS, OD_{680} and F_V/F_m values. Calibration replicates were performed
312 once the steady state was reached i.e. VSS, OD_{680} and F_V/F_m values were similar between replicates. The
313 calibration procedures included a control reactor, i.e. the nitrite standard solution was not added but the
314 reactor was operated in the same conditions. The inihibition effect was the difference between photosyntetic
315 activity in the control reactor and the inhibition assays. Calibration tests were attempted at a maximum of
316 2.5 h to ensure similar biomass from the semi-continuous reactor. The calibration setup scheme is shown
317 in Fig. 1B.

318 [FIGURE 1 NEAR HERE]

319 Figure 1. Scheme of the experimental setups for (A) inhibition and (B) calibration tests. Legend: 1 = HRAP; 2 = Industrial grade PC;
320 3 = relay box; 4 = multiparametric consort; 5 = on-off electro-valve; 6 = acid/base solution; 7 = pure CO_2 gas bullet; 8 = air
321 compressor; 9 = light source; 10 = reactor; 11 = DO, pH and temperature probes

322 2.2.2.3. Mathematical models

323 Two inhibition models were chosen to reproduce the effect of NO_2/FNA on photosynthesis: the non-
324 competitive inhibition model (Eq. 7) used to evaluated TNO_2 inhibition in nitrifying activity models (Claros et
325 al., 2013) and a sigmoidal logistic curve, or Hill-type model (Eq. 8) used to describe dose-response curve
326 (Prinz, 2010).

$$\frac{v}{v_{MAX}} = \frac{K_I}{K_I + I} \quad \text{Eq.7}$$

$$\frac{v}{v_{MAX}} = \frac{K_I^n}{K_I^n + I^n} \quad \text{Eq.8}$$

where v/v_{MAX} is the relative photosynthesis activity rate, v_{MAX} is the non-inhibited photosynthesis activity rate, K_I is the 50% inhibitor concentration, and n is the Hill coefficient.

The minimum residual sum of squared errors was used to match the experimental data with both inhibition models. The R-squared (R^2) was calculated to evaluate goodness of fit.

2.3. Analytical methods

Ammonium (NH_4-N), nitrite (NO_2-N) and nitrate (NO_3-N) concentrations were analyzed on an automatic analyzer (Smartchem 200, WestcoScientific Instruments, Westco) according to the Standard Methods (APHA-AWWA-WPCF, 2012): 4500-NH3-G, 4500-NO2-B and 4500-NO3-H, respectively. Total suspended solids (TSS), VSS and sCOD were analyzed according 2540-TSS-D, VSS 2540-VSS-E and 5220-COD-D, respectively (APHA-AWWA-WPCF, 2012).

The OD_{680} and fluorescence parameters were measured by a portable AquaPen-C AP-C 100 (Photon Systems Instruments, Czech Republic).

2.4. Statistical analysis

As previously mentioned, all the experiments were performed in triplicate to calculate the mean values and standard deviation. The relationships between the photosynthetic parameters and the NO_2-N or FNA concentrations were determined by an ANOVA analysis on STATGRAPHICS Centurion XVI.I software. P-values < 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. NO_2/FNA inhibition on microalgae photosynthesis.

To determine the true inhibitory species, concentrations of TNO_2 and pH values were systematically varied in batch tests. It was expected that if NO_2-N was the inhibitor, increasing it would reduce OPR_{NET} regardless of the pH value. If the OPR_{NET} reduction were only noted at pH 4.5, then the inhibiting species would be the FNA.

Figs. 2A and 2B show OPR_{NET} for 5 days at different TNO_2 concentration levels evaluated in the experiments carried out at pH 4.5 and 8, respectively. DO dynamic mass balance was attributed to photosynthesis and microalgae respiration since the nitrifying and heterotrophic bacteria activity was negligible.

Microalgae activity was normalized (i.e. expressed as a percentage of the maximum OPR_{NET}) to compare the impact on microalgae metabolism of different NO_2/FNA ratios at different pH values. At pH 8, the chemical equilibrium completely shifted towards the NO^-_2 species, i.e. TNO_2 consisted of 100% NO_2-N species. The second set of three batch reactors was run at a fixed pH value of 4.5, with a species distribution of 95.86% and 4.14% of NO_2-N and FNA at 25 ºC, respectively. The FNA concentration studied (Table 1) was higher than that achieved in the experimental setups described in González-Camejo et al. (2020), in which inhibition was already observed. Although FNA concentrations were low (< 5 g N m^{-3}), even lower concentrations that inhibit different bacterial taxonomic groups can be found in the literature (Blackburne et al., 2007; Claros et al., 2013; Pijuan et al., 2010).

Fig. 2 shows a continuous reduction of OPR_{NET} in both sets of experiments compared to the control reactor (p-value < 0.05). Photosynthesis was inhibited throughout the 5 experimental days at the concentrations tested (Table 1) in both sets of experiments. The results obtained in the two sets of three-batch assays confirmed that the microalgal activity inhibition was a consequence of NO_2-N accumulation and not of FNA, since increasing NO_2-N concentration significantly reduced (p-value < 0.05 obtained by ANOVA test) the ORP_{NET} regardless of the pH value. Understanding and quantifying the inhibition of photosynthetic activity by NO_2-N is of particular interest in wastewater treatments based on microalgal and bacterial consortia, since NO_2-N accumulation in the range of 15 to 50 g N m^{-3} has been observed in these systems (Akizuki et al., 2019; González-Camejo et al., 2020b, 2017; Van Den Hende et al., 2016) so that, the specific effects of NO_2-N on microalgae photosynthesis were investigated using ETR, NPQ and JIP-test analyses.

[FIGURE 2 NEAR HERE]

Figure 2. Normalized OPR_{NET} at a pH value of (A) 4.5 and (B) 8 for different concentration of TNO_2 after 5 experimental days: ●, 0 g N m^{-3}; ■, 25 g N m^{-3}; and ◄, 50 g m^{-3}.

3.2. NO_2-N inhibition mechanism

3.2.1. NO_2-N impact on ETR and NPQ

The impact of NO_2-N on photosynthesis activity was analyzed from the data obtained from assays performed at pH 8 rather than 4.5 due to: (I) the mean pH value in microalgae-bacteria systems dominated by microalgae and nitrifying bacteria is approximately 7.5 (daily-average) and tends towards alkaline pH values during the day-time (Mantovani et al., 2020); and (II) the optimal pH of microalgae, including species of the *Scenedesmus* genus, ranges from 7 to 9 (Barceló-Villalobos et al., 2019; Rossi et al., 2020a). The results obtained from photosynthetic activity at pH 8 described NO_2-N inhibition under typical operating conditions in a microalgae-bacteria system and favorable environmental conditions for *Scenedesmus*. All the results described below were compared with the values obtained in the control reactor.

The ETR curve experimental profile and NPQ values obtained are shown in Fig. 3. The ETR curves were found to be highly dependent on NO_2-N concentration (Fig. 3A). The microalgae α, ETR_{MAX} and E_K values were negatively affected at the two concentrations tested after 5 experimental days in both sets (Table 3). However, these parameters showed different inhibition patterns. The α values showed slight reduction from B8_25 to B8_50 (3%), and decrease to a maximum of 13% (p-value < 0.05) from the control reactor to B8_50. ETR_{MAX} and E_K declined significantly with a reduction of up to 35 and 25% (p-value < 0.05) in B8_50, respectively. The ETR curve response matched the type 1 photoinhibition described by Proctor and Bates (2018). At low irradiance ETR response increased with light intensity, but at higher irradiance (well below 1000 µmol m^{-2} s^{-1}) the ETR measurements sharply declined. Type 1 photoinhibition was related to an increase in NPQ on microalgae, as shown in Table 3. The influence of NO_2-N approximately doubled and tripled the NPQ values in B8_25 and B8_50, increasing by up to 276% (p-value < 0.05) in B8_50 with respect to the control reactor. According to Hernández-Zamora et al. (2014) this pattern (reduced ETR curve parameters and higher NPQ values) results from partial inhibition of the primary photosynthetic process (light-dependent reactions), reducing the capacity to process light energy. Light-driven reactions regulate the excitation energy harvested by the antenna complex, so that it can be balanced by the electron transport chain and synthesis of ATP and NADPH to reduce the potentially damaging effect on PS II by an excess of excitation energy, which can lead to a photooxidation process. On the other hand, the higher NPQ values

can be interpreted as an increase in thermal energy release due to the operation of the xanthophyll cycle (photoprotection mechanism which consists of reversible and rapid conversion from pigment diadinoxanthin to energy-dissipating form diatoxanthin), or gradually reversible damage to the microalgae's photosynthetic apparatus (Jiang et al., 2008; Roach and Krieger-Liszkay, 2014; Schulze and Caldwell, 2012).

[FIGURE 3 NEAR HERE]

Figure 3. Effect after 5 days of exposure on TNO_2 (A) ETR, (B) raw OJIP transient and (C) relative variable fluorescence and kinetic difference of V_{OP} at pH value of 8 for different concentrations of TNO_2: ●, 0 g N m^{-3}; ■, 25 g N m^{-3}; and ▲, 50 g N m^{-3}.

3.2.2. NO_2-N impact on JIP-test

According to the ETR parameters and NPQ values, NO_2-N had a significant effect on the electron transport chain and on the light energy harvesting efficiency. However, none of these provided accurate data on the effect of NO_2-N on the photochemical reactions. The polyphasic rise of *Chl a* fluorescence transients were analyzed to determine the specific inhibition mechanism (Figs. 3B and 3C). OJIP transients account for the sequential reduction of the electron acceptor pools of PS II. The fluorescence transient starts at phase O (at 50 μs), passes through two intermediate phases J and I (at 2 and 30 ms, respectively) and reaches the maximum fluorescence in phase P (Strasser et al., 2004). The raw fluorescence rise kinetic OJIP curves of batch reactors operated at pH 8 after 5 experimental days are shown in Fig. 3B. The microalgae cells exhibited a raw polyphasic OJIP rise kinetic in B8_0, similar to that described by Markou et al. (2016) and Perales-Vela et al. (2007). After 5 days of microalgae growth with NO_2-N, the raw transients gradually decreased when NO_2-N concentration increased with slightly lower (p-value < 0.05) variable fluorescence values than the control reactor ($\mathrm{F_v} = \mathrm{F_m} - \mathrm{F_0}$; where F_m is the maximal fluorescence intensity and F_0 is the fluorescence intensity at 50 μs) was detected in the O- J- I- P-step in B8_25, in contrast to the sharp decrease in F_v in B8_50 (p-value < 0.05). The gradual fall of the fluorescence rise kinetic OJIP curves along with NO_2-N concentration levels resulted in the accumulation of oxidized P_{680}^{+} (PS II reaction center) (Ji et al., 2018). P_{680}^{+} accumulates when the electron transport chain after the first electron acceptor Q_A is inhibited and the oxygen-evolving complex (OEC) cannot provide electrons to PS II and reduce PQ, which is consistent with the ETR results (Fig. 3A). Reduction of ETR and OJIP transients in nitrite-stressed reactors

could suggest that microalgae cells did not have the potential to grow due to NO_2-N (Zhang et al., 2010; Zhao et al., 2008).

A fluorescence normalization (between F_o and F_m) was performed to better evaluate the events or discrepancies reflected in the O-J, J-I and I-P phases, to obtain the relative variable fluorescence kinetics at any time, V_{OP}, (Eq. 5) and a kinetic profile of differences ΔV_{OP} (Eq. 6) (Gomes et al., 2012). V_{OP} variation between control and B8_25 and B8_50 showed a slight reduction in the overall OJIP curve transient with increasing inhibitor concentration, but not statistical changes (p-value < 0.05) in fluorescence peaks (O, J, I, P) were detected. However, B8_25 was characterized by a reduction of ΔV_{OP} values only in the I-P phase, while in B5_50, the NO_2-N concentration considerably reduced both the O-J and I-P phases from the OJIP curve. The OJIP curve phases represent different reduction processes of the electron transport chain (Gomes et al., 2012). The first phase, O-J, is related to the photochemical reduction of the primary acceptor, Q_A, in PS II reaction centers. The J-I phase reflects the reduction of the PQ (plastoquinone) pool, while the last phase (I-P) represents the reduction of both plastocyanin and P_{700}^{+} in photosystem I (PS I). I-P phase reduction was visible in both batch reactors, but the decrease in this specific OJIP transient in B8_50 was more pronounced. The I-P phase decreased with increasing NO_2-N supply, indicating that NO_2-N showed a fractional reduction of PS I final electron acceptors ($NADP^+$) (Roach and Krieger-Liszkay, 2014). The results suggest that the higher the NO_2-N concentration, the more limited the NADPH oxidation for carbon assimilation (Roach and Krieger-Liszkay, 2014). A reduction of transient J-I phase was only found in B8_50. Deactivation of the donor side of PS II has a negative effect on the J-I phase (Schreiber and Neubauer 1987), which is related to the reduction of the PQ-pool, electrons being necessary for this process. The required electrons are only produced when there is an intact and active manganese cluster. A loss of the J-I phase at 50 g N m^{-3} of NO_2-N suggested that inhibitor stress destroyed the oxygen-evolving complex due to a loss of manganese cluster activity on passing from 25 to 50 g N m^{-3} of NO_2-N.

JIP-test parameters from the OJIP kinetic curve were calculated and analyzed in detail. The ratio of variable to maximum fluorescence (F_V/F_M) represents the light absorbed by PS II to reduce Q_A, i.e. F_V/F_M is the maximum quantum efficiency of PS II. The value of F_V/F_M generally varies slightly under non-stressed conditions and is not influenced by microalgae species or operating conditions. However, marked

455 fluctuations in F_V/F_M values under stress have also been reported (Ji et al., 2018), making it a useful indicator
456 of photosynthesis efficiency and stress. The performance index (PI) describes the potential for energy
457 conservation of photons absorbed by PS II to reduce Q_A (PI_{ABS}) (Ji et al., 2018) and the reduction of the final
458 PS I acceptor (PI_{TOT}) (Strasser et al., 2010). Significant differences were found among F_V/F_M values (p-value
459 < 0.05) and both PI values (p-value < 0.05) for B8_25 and B8_50 compared to the control reactor (Table 3).
460 F_V/F_M, PI_{ABS} and PI_{TOT} values of microalgae grown in B8_25 were 13.49, 32.70 and 63.16% lower (p-value
461 < 0.05), respectively, than the values obtained in the control reactor. Microalgae cells subjected to 50 g N
462 m^{-3} of NO_2-N showed a 22.23, 51.97 and 85.40% reduction (p-value < 0.05) in F_V/F_M, PI_{ABS} and PI_{TOT},
463 respectively. The drop in F_V/F_M, PI_{ABS} and PI_{TOT} values in reactors operated with NO_2-N indicated that the
464 RC of PS II were damaged, the light-dependent reactions were inhibited and the electron transport chain
465 was hindered, as reported in other studies (Ji et al., 2018). PI_{ABS} and PI_{TOT} were more sensitive than F_V/F_M
466 to NO_2-N stress. This is consistent with previously published results (Charalampous et al., 2019; Ji et al.,
467 2018; Sun et al., 2020) in which PIs were considered the most sensitive transient parameter for indicating
468 inhibition processes.
469 The specific energy fluxes ABS/RC, TR_0/RC and DI_0/RC showed that for both B8_25 and B8_50, NO_2-N
470 had a negative effect on photosynthetic activity. As shown in Table 3 increasing NO_2-N concentration raised
471 the specific energy flux parameters. The ABS/RC parameter is the effective antenna size per active reaction
472 center, while TR_0/RC is the trapped energy flux per active reaction center. When microalgae were grown
473 with 25 g N m^{-3} and 50 g N m^{-3} of NO_2-N, ABS/RC values were 19.20% and 54.47% higher than for the
474 control reactor, respectively, and TR_0/RC values were 13.34% and 21.70% higher than B8_0, both indicating
475 a significant inhibitory effect (p-value < 0.05). On the other hand, DI_0/RC is the dissipated energy flux per
476 active reaction center. Comparing ABS/RC and TR_0/RC with DI_0/RC, the former showed an overall sluggish
477 upward tendency, while DI_0/RC rose sharply, especially in the highest NO_2-N concentrations, 31.81% and
478 123.75% in B8_25 and B8_50, respectively (p-value < 0.05). It is worth mentioning that the term RCs refers
479 only to the Q_A-reducing RCs (Strasser et al., 2004), so that the fact that the specific energy fluxes increased
480 with a higher NO_2-N concentration suggests that the Q_A-reducing RCs were negatively affected due to NO_2-
481 N damage. The ABS/RC ratio thus increased by the deactivation of some active RCs, resulting in an
482 intracellular accumulation of energy (Ji et al., 2018) and raised DI_0/RC. The trend of increasing TR_0/RC

implied that all the Q_A was reduced but was not able to reoxidize due to NO_2-N damage, i.e., reoxidation of Q_A^- was inhibited and could not efficiently transfer electrons to Q_B (Mehta et al., 2010). The higher DI_0/RC was the microalgae cells' self-protective mechanism against the damage by the excess of intracellular energy, so that the absorbed energy was dissipated instead of being used to reduce Q_A (Ji et al., 2018). This conclusion is supported by the fact that NPQ values increase on exposure to NO_2-N (when photochemistry is partially inhibited, energy dissipation through NPQ is expected to increase) (Juneau et al., 2002).

Adding NO_2-N to a microalgae culture reduces the flux ratios of the photochemistry values, φ_{Po}, φ_{Eo} and ψ_{Eo} (Table 3). φ_{Po} is the excitation energy capture probability of PS II, φ_{Eo} is the quantum yield for PQ pool reduction and ψ_{Eo} is defined as the probability electron transfer from Q_A in PS II to the PQ pool (Todorenko et al., 2021). φ_{Po} values in B8_25 and B8_50 were 5% and 21.24%, respectively, lower than the results achieved in B8_0 (p-value < 0.05). The decrease in φ_{Po} indicated an inhibited light reaction. The reduction efficiency of light photochemical reactions (φ_{Po}) led to higher energy dissipation (Chen et al., 2016), this being consistent with the trends described in NPQ and DI_0/RC. The flux ratio φ_{Eo} showed a reduction of 11.57% and 29.44% in B8_25 and B8_50, respectively, while ψ_{Eo} reduction was 6.92% and 10.40% compared to the control microalgae culture (p-value < 0.05). The downward trend of φ_{Eo} and ψ_{Eo} agreed with the changes in the F_V/F_M and PI_{ABS} parameters with a higher NO_2-N concentration. The inhibition effect of NO_2-N can reduce light absorption, hinder electron transfer efficiency and reduce the maximum electron microalgae's transfer yield.

Unlike the three flux ratios described above, φ_{Do}, δ_{Ro} and φ_{Ro} are associated with non-photochemical processes (dos Santos Farias et al., 2019). φ_{Do} is the quantum yield of energy dissipation (Sun et al., 2020), δ_{Ro} is the electron transfer probability beyond the PQ pool and φ_{Ro} is the quantum yield of electron acceptor reduction in PS I (Chen et al., 2016). φ_{Do} was 13.10% and 48.24% in B8_25 and B8_50, respectively (p-value < 0.05) higher than in the control reactor. The higher heat dissipation energy indicated that the light energy utilization efficiency decrease (φ_{Po}). δ_{Ro} fell by 15.76% and 39.40%, while φ_{Ro} was reduced by 13.10% and 48.24% in B8_25 and B8_50, respectively, compared to R8_0 (p-value < 0.05). The studied NO_2-N concentrations reduced the probability of an electron being transported to the PS I final electron

acceptor (δ_{Ro}) and reduced the quantum yield for the reduction of the final electron acceptors on the acceptor side of PS I (φ_{Ro}).

The parameters directly derived from the transient OJIP curve, V_J, M_o and S_M, represent the relative variable fluorescence at peak J (Sun et al., 2020), the initial slope of the OJIP transient normalized on the maximal F_V and the electron numbers that pass through the electron transport chain, respectively (Franić et al., 2018). V_J increased by 143.27% and 215.63%, while values of M_o were 176.07% and 284.62% higher in B8_25 and B8_50, respectively, compared to the control reactor (p-value < 0.05). The increase of V_J and M_o as NO_2-N exposure rose (p-value < 0.05) reflected the accumulation of Q_A^-, while M_o indicated that the net rate of the reaction center closure was mainly in the oxidized state (Matorin et al., 2013). On the other hand, the reduced S_M values with higher NO_2-N levels (p-value < 0.05) suggested that maximum fluorescence could be reached quicker, as fewer electrons were needed to reduce PS II electron acceptors, indicating NO_2-N's inhibitory effect (Franić et al., 2018).

Up to now, the effects of NO_2-N on ETR, NPQ, polyphasic OJIP rise kinetics and transient parameters have not been reported. However, it can be concluded that the parameters' behavior described is similar to the results reported by other authors for microalgae exposed to different toxic compounds, such as free ammonia (Markou et al., 2016), herbicides (Magnusson et al., 2008; Sun et al., 2020), or heavy metals (Perales-Vela et al., 2007).

Table 3. Photosynthetic parameters related to the electron transport chain and the JIP-test affected by NO_2-N concentration in batch reactors operated at pH 8.

Photosynthetic parameters	B8_0 (control)			B8_25			B8_50		
α (electrons/photon)	0.5241	±	0.0004	0.4715	±	0.0004	0.458	±	0.006
E_K (μmol m^{-2} s^{-1})	219	±	10	203.6	±	0.2	164	±	2
ETR_{MAX} (μmol m^{-2} s^{-1})	115	±	5	96	±	2	75	±	3
NPQ	0.227	±	0.04	0.45	±	0.04	0.85	±	0.08
V_J	0.046	±	0.002	0.112	±	0.002	0.145	±	0.002
F_V/F_M	0.739	±	0.009	0.639	±	0.006	0.57	±	0.03
M_o	0.078	±	0.002	0.215	±	0.005	0.300	±	0.003
S_M	384	±	13	329	±	18	267	±	23
ABS/RC	2.50	±	0.13	2.98	±	0.02	3.86	±	0.07
TR_o/RC	1.70	±	0.07	1.922	±	0.007	2.06	±	0.02

DI_o/RC	0.80	±	0.06	1.06	±	0.02	1.80	±	0.05
φ_{Po}	0.679	±	0.004	0.645	±	0.002	0.535	±	0.004
φ_{Eo}	0.648	±	0.003	0.573	±	0.003	0.457	±	0.003
ψ_{Eo}	0.954	±	0.002	0.888	±	0.002	0.855	±	0.002
φ_{Do}	0.314	±	0.006	0.355	±	0.004	0.465	±	0.004
δ_{Ro}	0.69	±	0.02	0.58	±	0.05	0.415	±	0.004
φ_{Ro}	0.4381	±	0.0114	0.30	±	0.03	0.189	±	0.002
PI_{ABS}	11.99	±	0.98	8.1	±	0.4	5.8	±	0.7
PI_{TOTAL}	9.37	±	1.13	3.5	±	0.3	1.4	±	0.2

529

530 3.2.3. Overall NO_2-N effects on photosynthesis

531 Exposure to NO_2-N produced a partial inhibition of the primary photosynthetic process (F_V/F_M, φ_{Po}, PI_{ABS})
532 mainly due to hindrance of the electron transport chain (ETR curve). Under non-stress conditions, i.e. no
533 NO_2-N accumulation (Fig. 4A), the excitation energy obtained from light is absorbed by the reaction center
534 (RC) of PS II, (P_{680}). The excited P_{680} is oxidized by releasing an electron which is transported across the
535 membrane to the first electron acceptor, Q_A, then the electron passes to the second acceptor, Q_B, and finally
536 reduces PQ to PQH_2. Electrons passing through the electron transport chain lose energy that is used to
537 pump H+ ions by complex Cytochrome b6f. These concentrated ions store potential energy forming an
538 electrochemical gradient. H^+ ions "slide" down their concentration gradient. As they flow, the ion
539 channel/enzyme ATP synthase uses its energy to produce ATP. At the end of the chain, electrons bind H^+
540 ions to $NADP^+$ to produce NADPH (non-cyclic photophosphorylation). The excited electrons can take an
541 alternative path called cyclic electron flow, which only is involved the PS I. Electrons flow from the electron
542 acceptor ferredoxin to Cytochrome b6f, with ATP being the only product (cyclic photophosphorylation).
543 However, under NO_2-N stress conditions (Fig. 4B), the electron transport chain between PS II and PS I is
544 hindered and the probability of an electron being transported to PS I is reduced (I-P phase, PI_{TOT}, δ_{Ro}, φ_{Ro}
545 and S_M). The reduced Q_A is not able to reoxidize and cannot efficiently transfer electrons to Q_B (phase O-J
546 and TR_0/RC), accumulating P_{680}^+ (raw kinetic OJIP curves, V_J, M_o) and PQ (φ_{Eo} and ψ_{Eo}). Therefore, the
547 P_{680}^+ cannot be re-reduced and the oxidative splitting of water into four protons and molecular oxygen is
548 limited (OPR_{NET}). ATP and NADPH production are negatively affected, reducing sugar building in the second
549 stage of photosynthesis.

The preceding energy of excited electrons are accumulated and damages RCs of PS II (ABS/RC). As a self-protective mechanism, the excess of intracellular energy is dissipated as heat (NPQ, DI_0/RC and φ_{Do}).

[FIGURE 4 NEAR HERE]

Figure 4. Simplified and adapted Z-scheme of the photosynthetic electron transport chain under non-stress conditions (A) and NO_2-N-stress conditions. The scheme shows the redox reactions needed to transfer one electron from H_2O to NADPH and produce ATP. Abbreviations: OEC = oxygen-evolving complex; RC = reaction center; Q_A = PSII primary quinone acceptor; Q_B = PSII secondary quinone acceptor; cytb6f = complex cytochrome b6f; LHC II = light-harvesting complex of Photosystem II; LHC I = light-harvesting complex of Photosystem I; *Chl* a II = *Chlorophyll a* of Photosystem II; and *Chl a* I = *Chlorophyll a* of Photosystem I. The dotted straight arrow shows the pathway of non-cyclic and cyclic photophosphorylation.

3.3. Calibration of NO_2-N inhibition

The results described above show that the inhibition of microalgae activity is a consequence of NO_2-N concentration. A total of ten experiments were carried out at different NO_2-N concentrations to determine and calibrate the inhibitory effect on photosynthesis activity. The sets of calibration assays were carried out on different days. To properly compare and calibrate the effect of NO_2-N, inhibitory effects were thus obtained as the difference between photosynthesis activity in the control and NO_2-N stressed microalgae assays. Photosynthetic parameters were normalized and expressed as the percentage of the maximum value. Fig. 5 shows the photosynthetic activity parameters in terms of percentage versus total NO_2-N concentrations. The trend of OPR_{NET}, NPQ, ETR_{MAX} (the most sensitive ETR parameter) and PI_{ABS} are shown in Figs. 5A, B, C and D, respectively. From the OJIP transient parameters, only PI_{ABS} was used to calibrate NO_2-N damage since it was one of the most sensitive parameters for NO_2-N damage and in the literature it is commonly used to identify inhibition effects (Chen et al., 2016; Ji et al., 2018; Sun et al., 2020).

Experimental results were used to match the non-competitive inhibition model (Eq. 7) and the Hill-type model (Eq. 8). The latter can describe all the photosynthetic parameters with a high degree of accuracy (R^2 higher than 0.97, Table 4), while the non-competitive model was not able to accurately describe NPQ and PI_{ABS} values (R^2 less than 0.80). The variability in the results differed according to the parameters measured, as shown by the bandwidth of the 95% confidence limits (Figs. 5A, B, C and D). ETR (Fig. 5C) and PI_{ABS} (Fig. 5D) values were more accurate than OPR_{NET} (Fig. 5A) and NPQ (Fig. 5B).

For all experiments, the photosynthetic parameters followed a similar decreasing trend when increasing total NO_2-N concentration. The NO_2-N inhibition constants were determined by minimizing the root mean square error between model prediction (Hill-type model) and experimental data. Values of $K_{I,NO2}$ (Fig. 5E) and n_{NO2} (Fig. 5F) and the 95% confidence limits of each parameter are listed in Table 4. Values of $K_{I,NO2}$ ranged from 24.10 to 40.54 g NO_2-N m^{-3}, while n varied between 1.86 and 5.90. The least sensitive photosynthetic parameter was ETR_{MAX}, showing an inhibition constant of 40.54 g NO_2-N m^{-3}, while the remaining parameters are practically the same, ranging from 24.10 and 26.99 g NO_2-N m^{-3}. The OPR_{NET} trend could be matched with a non-competitive inhibition model with a good degree of accuracy, as the Hill constant value is near to 1 (1.86) and 0.95 of R^2. Regarding only OPR_{NET} values, there was little difference between the two models. To reduce computational cost, the non-competitive inhibition model is preferable because a similar prediction can be obtained with a single parameter. However, the Hill-type model was selected to reproduce NO_2-N inhibition since it matches the trend of the 4 photosynthetic parameters, while the non-competitive inhibition model only reproduces the OPR_{NET} results.

[FIGURE 5 NEAR HERE]

Figure 5. Effect of NO_2-N on microalgae. Hill-type model and non-competitive inhibition model fit for normalized parameters: (A) OPR_{NET}, (B) NPQ, (C) ETR_{MAX} and (D) $PI_{ABS;}$ and estimated model parameters: (D) half inhibition constant (K_I) and (E) Hill constant. Shaded areas and error bars represent the 95% confidence intervals.

Up to now, inhibition parameters for microalgae related to NO_2-N toxicity effects have not been reported, so that direct comparison and analysis with previous works is not possible.

Table 4. Selection criteria and Hill-type model calibrated parameters. Average data of each parameter are reported as mean value ± standard deviation; and 95% confidence intervals on calibrated parameters are reported in square brackets.

Parameter calibrated	R^2	K_I					n				
OPR_{NET} (g O_2 m^{-3} d^{-1})	0.9486	23.7	±	1.2	[21.89	25.54]	1.82	±	0.14	[1.59	2.04]
NPQ	0.9742	26.36	±	1.1	[24.62	28.10]	5.3	±	0.5	[4.44	6.08]
ETR_{MAX} (µmol m^{-2} s^{-1})	0.9873	39	±	2	[35.77	41.25]	2.4	±	0.4	[1.79	2.94]
PI_{ABS}	0.9915	26.5	±	0.4	[25.81	27.71]	4.2	±	0.2	[3.91	4.45]

According to the calibrated microalgae model by Aparicio et al. (2022), the following kinetic expressions are proposed to represent the microalgae growth rate in a culture medium with ammonium and soluble phosphorus, as nitrogen and phosphorus sources, respectively, and with NO_2-N production by nitrifying bacteria (Eq.9).

$$\mu_{ALG} \cdot \frac{S_{Ig,C}}{K_{Ig,C} + S_{Ig,C}} \cdot \frac{S_{NHX}}{K_{NHX} + S_{NHX}} \cdot \frac{S_{PO4}}{K_{PO4} + S_{PO4}} \cdot \frac{K_{I,NO2}{}^{n_{NO2}}}{K_{I,NO2}{}^{n_{NO2}} + S_{NO2}{}^{n_{NO2}}} \cdot X_{ALG} \cdot f_L \cdot f_{pH} \cdot f_T \qquad \text{Eq. 9}$$

where μ_{ALG} is the maximum growth rate for microalgae (d^{-1}); $S_{Ig,C}$ is the total inorganic carbon (mol C m^{-3}); $K_{Ig,C}$ is the half saturation parameter for inorganic carbon (mol C m^{-3}); S_{NHx} is the ammonium plus free ammonia nitrogen (g N m^{-3}); K_{NHX} is the half saturation parameter for the ammonium plus free ammonia nitrogen (g N m^{-3}); S_{PO4} is the total soluble inorganic phosphorus (g P m^{-3}); K_{PO4} is the half saturation parameter for the total soluble inorganic phosphorus (g P m^{-3}); S_{NO2} is nitrite nitrogen (g N m^{-3}); $K_{I,NO2}$ is the inhibitor concentration that produce 50% inhibition (g N m^{-3}); n_{NO2} is the Hill coefficient; X_{ALG} is the microalgae biomass (g COD m^{-3}), f_L is the light factor; f_{pH} is the pH factor and f_T is the thermic factor.

As mathematical modeling of microalgae is usually based on oxygen production (Rossi et al., 2020a; Sánchez Zurano et al., 2021; Solimeno et al., 2017), it is recommended to use the $K_{I,NO2}$ value obtained in OPR_{NET} assays to introduce the NO_2-N inhibition process.

González-Camejo et al. (2020) found that a short 30-min exposure to NO_2-N did not have significant effect on the photosynthetic activity of the indigenous microalgae *Chlorella*. In this study, the calibration procedure consisted of a 20-min exposure to NO_2-N and significant results of the toxic effect on photosynthesis were achieved. Short-term exposure photosynthesis inhibition was evaluated only by the OPR_{NET} trend in the study conducted by González-Camejo et al. (2020), while in the present study inhibitory effects were analyzed through the combination of OPR_{NET} and fluorescence parameters which provided more specific results. A microalgae-bacteria culture was obtained from outdoor pilot-scale reactors in both studies. The ever-changing environmental and operating conditions promote community shift between different biological communities, for example changes in the dominance of *Scenedesmus* over the *Chlorella* genus. Comparing both studies, *Scenedesmus* could be more sensitive to the NO_2-N inhibitory effect than *Chlorella*, as

624 reflected in a reduction in OPR_{NET} when exposing microalgal cells to different NO_2-N concentrations. The
625 inhibitory effect of NO_2-N can be species-dependent.
626 Microalgal model, to which NO_2-N inhibition of microalgae growth has been added, was validated with
627 different structures of microalgae communities (8% Chlorella and 92% Scenedesmus; 56% Chlorella and
628 44% Scenedesmus; 87% Chlorella and 13% Scenedesmus; and 96% Chlorella and 4% Scenedesmus)
629 (Aparicio et al., 2022; Viruela et al., 2021). However, calibration procedure was performed with a community
630 structure dominated by different *Scenedesmus* genera. Comparing with results obtained by González-
631 Camejo et al. (2020), it was deduced that different microalgae show different response and sensitivity to
632 NO_2-N stress. To depict the actual condition in the biological system, NO_2-N should therefore be validated
633 with operating periods that combine different community structures. Future work is aimed at developing a
634 comprehensive microalgae-bacteria model, including NO_2-N inhibition and its validation with different
635 microalgae culture technologies (membrane HRAP and membrane photobioreactor), different input
636 wastewater streams and community structures.
637
638 3.4. NO_2-N scenarios in microalgae-based wastewater treatment
639 The use of estimated parameters for NO_2-N can be particularly useful to assess the extent of NO_2-N
640 inhibition under common operating and environmental conditions of microalgae and bacterial cultivation. For
641 example, NO_2-N can accumulate from variations of light intensity, temperature and pH that promote AOB
642 over NOB activity (partial nitrification). Although the nitrifying bacteria can be inhibited by high light intensity
643 (S Akizuki et al., 2019), Akizuki et al. (2020) and Vergara et al. (2016) suggested NOB had a higher light
644 sensitivity. Vergara et al. (2016) reported that NOB photoinhibition happened under a continuous illumination
645 of a 250 μmol m^{-2} s^{-1} light intensity, while NO_2-N continued to accumulate at intensities above 500 μmol m^{-2} s^{-1}. Nitrification is more strongly regulated by average daily light intensity than instantaneous incident light
647 (Akizuki et al., 2020). The average daily light intensity recorded in the MHRAP pilot plant operated in this
648 study ranged from 158 ± 60 to 301 ± 48 μmol m^{-2} s^{-1} in winter and summer respectively, and may promote
649 NOB photoinhibition in summer. Temperature is also a key environmental parameter that can influence both
650 NO_2-N and NO_3-N production rates. Partial nitrification and NO_2-N accumulation were generally promoted
651 at temperatures between 20 – 35 ºC, at which, the specific AOB growth rate is higher than that of NOB (Gao

et al., 2010; Kim et al., 2006). The culture temperature in MHRAP pilot plant was 17 ± 2 and 29.1 ± 0.8 ºC in winter and summer respectively, so that warm temperatures can influence nitrite concentration and inhibit photosynthesis.

pH values commonly increase during daylight hours due to photosynthesis. For example, the average pH value of HRAP was 7.5 ± 0.7 in winter, while in summer, it rose to 8.2 ± 0.3 when photosynthesis was higher. The optimum pH range for AOB and NOB ranges 8.2 – 8.4 and 7.7 – 7.9 respectively (Park et al., 2007). NOB are more sensitive to alkaline pH, which in microalgae cultures is usually recorded in summer.

The most favorable scenario for NO_2-N accumulation was in summer, with daily average light above 250 µmol m^{-2} s^{-1} and culture temperature and pH between 20 – 35 ºC and 8.2 – 8.4, respectively. During daylight, NO_2-N oxidation to NO_3-N was inhibited by increased light intensity, temperature and pH above NOB optimal range, resulting in an NO_2-N accumulation and inhibition of microalgal photosynthesis.

Nitrite concentration has been reported in the range of 15 to 50 g N m^{-3} in different microalgae-bacteria systems (Akizuki et al., 2019; González-Camejo et al., 2020b, 2017; Van Den Hende et al., 2016)

Different strategies can be used to limit and reduce the nitrite production rate in outdoor systems with ever-changing culture conditions. The simplest option is the complete inhibition of AOB activity using ATU, but this requires the continuous addition of chemical substances that involves environmental impact and cost, so that alternative operating strategies based on operational and environmental conditions are recommended. González-Camejo et al. (2020) and González-Camejo et al. (2019) propose temperature ranges and biomass retention times that promote: (I) microalgae growth over AOB and NOB growth; and (II) complete nitrification.

4. CONCLUSIONS

The effects of nitrite and free nitrous acid on microalgae photosynthesis were studied in batch reactors operated at pH 4.5 and 8. Nitrite, rather than the free nitrous acid, has an overall negative and rapid effect on photosynthesis, affecting simultaneously different sites of the photosynthetic apparatus. The present work suggests that the nitrite inhibition mechanism was based on reducing light absorption and hindering the electron transport chain. The Q_A was not able to transfer electrons to Q_B, so that the primary photosynthetic process was limited and the maximum electron transfer yield was also reduced.

679 The photosynthetic apparatus of microalgae responded to nitrite stress by increasing self-protective
680 mechanism to avoid damage by the excess of intracellular energy. The proposed Hill function was able
681 to accurately reproduce the inhibitory effect of nitrite on four photosynthetic parameters, OPR_{NET}, NPQ,
682 ETR_{MAX}, and PI_{ABS}. The $K_{I,NO2}$ resulted in 24.10, 26.99, 40.54, and 27.55 g N m^{-3} and the Hill parameter,
683 n, was 1.82, 5.3, 2.4 and 4.2 for the above four parameters, respectively. Comparing the results
684 obtained in this work with other scientific investigations, it is suggested that the inhibitory effect of nitrite
685 could be species-dependent, *Scenedesmus* being more sensitive than *Chlorella*.

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