Sensitivity of Photosynthesis to Warming in Two Similar Species of the Aquatic Angiosperm *Ruppia* from Tropical and Temperate Habitats

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**Abstract:** Climate change-related events, such as marine heatwaves, are increasing seawater temperatures, thereby putting pressure on marine biota. The cosmopolitan distribution and significant contribution to marine primary production by the genus *Ruppia* makes them interesting organisms to study thermal tolerance and local adaptation. In this study, we investigated the photosynthetic responses in *Ruppia* to the predicted future warming in two contrasting bioregions, temperate Sweden and tropical Thailand. Through DNA barcoding, specimens were determined to *Ruppia cirrhosa* for Sweden and *Ruppia maritima* for Thailand. Photosynthetic responses were assessed using pulse amplitude-modulated fluorometry, firstly in short time incubations at 18, 23, 28, and 33 °C in the Swedish set-up and 28, 33, 38, and 43 °C in the Thai set-up. Subsequent experiments were conducted to compare the short time effects to longer, five-day incubations in 28 °C for Swedish plants and 40 °C for Thai plants. Swedish *R. cirrhosa* displayed minor response, while Thai *R. maritima* was more sensitive to both direct and prolonged temperature stress with a drastic decrease in the photosynthetic parameters leading to mortality. The results indicate that in predicted warming scenarios, Swedish *R. cirrhosa* may sustain an efficient photosynthesis and potentially outcompete more heat-sensitive species. However, populations of the similar *R. maritima* in tropical environments may suffer a decline as their productivity will be highly reduced.

**Keywords:** marine heatwaves; PAM fluorometry; seagrass; $F_v/F_m$; NPQ; $F_v/F_o$; *Ruppia cirrhosa*; *Ruppia maritima*

1. Introduction

Global climate change is putting immense pressure on organisms and whole ecosystems [1–5]. Resilience and adaptation to such stress are key factors determining future biodiversity dispersal in terrestrial and marine environments. In the world’s oceans, average mean temperatures are predicted to increase by 1.3 °C before 2065 [1]. Even more alarming may be the acute effects of specific climate change-induced events such as marine heatwaves [2,3], especially influencing shallow coastal waters that get warmed rapidly and where temperatures may fluctuate greatly on a diel basis. Reportedly, such events have
caused mass mortality and shifts in ecosystem structure, threatening key habitats in the coastal seascape [2,4,5]. Seagrass meadows are important ecosystem engineers in coastal areas, supporting numerous ecosystem services. These include provision of nursing and living grounds for multiple marine organisms, climate change mitigation due to high CO₂ capture and sequestration, and water filtration by particle retention [6–8]. Due to increased anthropogenic impact, a worldwide seagrass decline has occurred [9,10], and these natural benefits are therefore under threat. Loss of certain seagrass species may in many areas promote colonization of more tolerant or opportunistic seagrass species or other submerged aquatic vegetation, hence altering the benthic plant community composition [11].

Generally, seagrasses respond with a linear increase in photosynthetic rates with temperature, until a threshold and concomitant tipping point is reached at high temperature [12–15]. These tipping points are highly species-specific and area dependent; for instance, temperate Zostera marina reaches its limit around 30 °C [12,14,16], Mediterranean Ruppia cirrhosa at approximately 36 °C [17], and tropical species may withhold photosynthetic rates up to 40 °C [15,18–21]. However, before those photosynthetic limits are reached, biomass loss and tissue degradation can already be encountered [20,22,23]. Negative or detrimental effects of the photosynthetic apparatus at high temperatures are often related to protein degradation, decreased protein synthesis, and loss of membrane stability [24]. Photosystem II (PSII) is the most heat sensitive of the two photosystems [25], where increased temperature may cause direct impairment of the oxygen-evolving complex [26] or indirect damage to the D1 protein caused by lipid peroxidation or accumulation of reactive oxygen species (ROS) [27–29]. Moreover, high temperature might stimulate photorespiration resulting in lower productivity, especially in conditions of high oxygen [30,31].

Insights from previous studies revealed diversified responses to warming depending on the plant history and their adaptive capacity to local environments [32–36]. Differing resistance to warming was observed in Zostera marina from northern and southern populations [37,38], whereas several studies shed light on heat stress responses of the Mediterranean seagrasses from different ambient temperatures [32–36]. Results from these investigations suggest higher thermotolerance and greater capability to acclimate to temperature changes in populations inhabiting warmer environments. Nevertheless, this observation is based on studies within the same bioregion. When considering a larger geographic scale, it is hypothesized that tropical marine species will be more sensitive to increasing temperature than temperate species. In addition to their evolutionary history in a relatively constant temperature, it is assumed that the upper temperature threshold of the tropical species are closer to the ambient temperature in their habitat [21,23,39–41]. Thus, warming events in tropical areas may push the organisms beyond their operating and survival limits. Studies across bioregions with different temperature regimes conducted using well-designed experimental approaches of ecological relevance are needed to provide supporting evidence for these assumptions. The existing literature thus far is concentrated on animal models, while studies on marine vegetation remain scarce [21,23,39–41].

The genus Ruppia comprises a group of aquatic angiosperm species, which are able to sexually reproduce in fresh, brackish, and marine environments [42,43]. This characteristic sets them aside from other seagrasses, which reproduce in marine waters only. Their ability to inhabit fresh and brackish environments suggests a broad range of environmental tolerance [42,43]. They are considered fast-growing pioneer species, which play an important role in shallow-water community dynamics and productivity [44]. In addition, their cosmopolitan distribution across different bioregions makes the genus Ruppia an interesting organism to study how local adaptation contributes to variability in thermal niches of aquatic plants.

The aim of the study was to explore the thermal sensitivity of Ruppia from two different bioregions, including R. cirrhosa from a temperate area and R. maritima from a tropical area. The two Ruppia species are closely related and functionally and morphologically similar [45,46], and each species contribute as an important primary producer in the shallow water of its respective region [44,47,48]. We focused on assessing their photosynthetic
responses to increased temperatures within a range occurring in their natural settings encompassing the warming scenarios. The results can provide valuable insights on the future effects of warming on submerged aquatic vegetation at genus and community levels in different bioregions.

2. Materials and Methods

2.1. Study Sites and Plant Material

As this study aims to capture the temperature response of *Ruppia* from two contrasting temperature regimes, we examined specimens from a temperate (Sweden) and a tropical (Thailand) bioregion (Figure 1A,B). The temperate species was identified as *R. cirrhosa* and the tropical one was identified as *R. maritima*. *Ruppia cirrhosa* is a representative species of the common submerged aquatic vegetation at the Swedish west coast [11], while *R. maritima* represents the only *Ruppia* species recorded in Thai waters [45,46]. The mean surface water temperatures (SST) in these regions normally fluctuates between −4 and 24 °C (Sweden) (Figure 1C) and 27 and 33 °C (Thailand) (Figure 1D) on a seasonal basis. However, in shallow coastal areas the temperatures may differ from the average SST. At the time of sampling, ambient temperature was 18 °C at the Swedish site and 28 °C for the Thai site.

The Swedish part of the study was conducted at Kristineberg Marine Research Station in Fiskebäckskil on the west coast of Sweden in July 2019 and the Thai part at the Coastal Oceanography and Climate Change Research Center at the Prince of Songkla University in January 2020. Specimens of *R. cirrhosa* were harvested from shallow soft bottoms at approximately 1.2 m depth in Fiskebäckskil, Sweden (58°14′34.8″ N 11°27′59.7″ E, Figure 1A), whereas specimens of *R. maritima* were collected from shallow areas of Pattani Bay, Thailand (6°53′41.4″ N 101°16′26.7″ E, Figure 1B). Seagrass specimens were instantly transported to the laboratory in buckets of seawater. Upon arrival, the plants were cleaned of sediment and visible epiphytes under running seawater. Specimens were then placed in aerated tanks connected to a seawater flow-through system and left five days for acclimation. Light was provided on a 12 h dark/12 h light scheme with fluorescent light tubes with PAR of approximately 100 μmol photons m⁻² s⁻¹ for Swedish *R. cirrhosa* and 200 μmol photons m⁻² s⁻¹ for Thai *R. maritima*, corresponding to the minimum saturating irradiance (Ek) levels derived from rapid light curves (RLCs, data not shown). The water temperature was maintained at 18 °C for the Swedish design and 28 °C for the Thai set-up. These levels were used as the ambient temperatures at the respective sampling periods. Salinity was 25 practical salinity units (psu) for the Swedish set-up and 30 psu for the Thai set-up (levels recorded at the sampling sites) and pH was 8.1. Please refer to Figure 1E for the experimental design and timeline for the measurements described below.

2.2. DNA Barcoding

2.2.1. Genomic DNA Extraction, Amplification, and Sequencing

As species identification using morphological features has been proven difficult for the genus *Ruppia*, determination was assisted using DNA barcoding. Plant material was homogenized with mortar and pestle in liquid nitrogen. The total genomic DNA was extracted following the manufacturer’s protocol of DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The quality and concentration of genomic DNA samples were determined by agarose gel electrophoresis and using the Thermo NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The amplification and sequence analysis of the internal transcribed spacer (ITS1-5.8S-ITS2) and ribulose-1,5-bisphosphate carboxylase/oxygenase N-methyltransferase (*rbcL*) regions were undertaken using the primer sets presented in Table 1. The PCR amplification was performed in a 50 μL reaction volume containing 10 μL of 5X Phusion™ HF Buffer, 1 μL of dNTP (10 μM), 1.5 μL of DMSO, 0.5 μL of Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA) (0.02 U/μL), 1 μL of each primer (10 mM), and 250 ng of template genomic DNA. The thermal cycle was initiated by 30 s of denaturation at 98 °C, followed by 35 cycles of 10 s of denaturation at
98 °C, 30 s of annealing at 55 °C and 20 s of elongation at 72 °C, and a final elongation of 5 min at 72 °C. The PCR products were gel-purified and submitted to sequencing at 1st BASE DNA Sequencing Services (Singapore).

Figure 1. (A,B) Locations of the collection sites, (C,D) sea surface temperature (SST) in Swedish and Thai waters recorded from 2018 to 2020, and (E) experimental design and timeline of the measurements. Sea surface temperatures were retrieved from GHRSST Level 4 MUR Global Foundation Sea Surface Temperature Analysis (v4.1) (https://podaac.jpl.nasa.gov/dataset/MUR-JPL-L4-GLOB-v4.1 accessed on 19 June 2021). The green arrows in C and D indicate the sampling periods in the two sites.
Table 1. The primer sets used in this study. Primer sequences, melting temperatures (Tm), and corresponding references are indicated.

| Primer  | F/R | Sequence 5’–3’                  | Tm  | Reference          |
|---------|-----|---------------------------------|-----|--------------------|
| ITS5    | F   | GGAAAGTAAAAGTCGTAACAAAGG        | 51.3| [49,50]            |
| ITS4    | R   | TCCTCCGTATTTGATATGC             | 52.1| [49,50]            |
| rbcL_F  | F   | ATGTCACCACAAACAGAGACTAAAGC      | 57.2| [49,50]            |
| rbcL_R  | R   | GAAACGGTCTCTCCAACGC             | 57.6| [49,50]            |

2.2.2. Phylogenetic Analysis

The ITS (ITS1-5.8S-ITS2) and rbcL sequences were analyzed using BLASTn version BLASTN 2.11.0+ (http://www.ncbi.nlm.nih.gov/blast accessed on 11 May 2021; RID 9JGJ93C016, RID9JH3TTV0016, RID 9JGHG8N013 and RID 9JH4N8VC013) and the MEGA software version X. The ITS (ITS1-5.8S-ITS2) and rbcL gene sequences of the Ruppia spp. reference strains; Accession No. AB728749.1 (R. cirrhosa ITS gene), MN958127.1 (R. drepanensis ITS gene), AB728734.1 (R. maritima ITS gene), JQ034337.1 (R. megacarpa ITS gene), JN113279.1 (R. maritima rbcL gene), NC051974.1 (R. brevipedunculata rbcL gene), JN113277.1 (R. cirrhosa rbcL gene), MN233650.1 (R. sinensis rbcL gene), and AB507891.2 (R. megacarpa rbcL gene) were selected from GenBank by subjecting the nucleotide sequences of ITS and rbcL using BLASTn. The datasets were divided into three sets; ITS (ITS1-5.8S-ITS2) dataset, rbcL dataset, and the combination of ITS (ITS1-5.8S-ITS2) and rbcL dataset. Multiple sequence alignment was achieved with ClustalW of MEGA X. Phylogenetic trees were constructed using the maximum likelihood method in MEGA X [51], based on the Kimura 2-parameter model. The maximum parsimony was performed by 1000 bootstrap replications [52].

2.3. Sensitivity of Photosynthesis to Warming

2.3.1. Photosynthetic Responses to a Series of Temperatures

Measurements were conducted in 3 mL airtight experimental chambers (model DWA1, Hansatech, King’s Lynn, UK). In each seawater-filled chamber, seven leaf segments (total width ~0.7 cm) with a length of 3 cm were placed next to each other in a U-shaped manner for effective light harvesting. Light was provided from the side using cold-light sources providing irradiance of approximately 100 µmol photons m⁻² s⁻¹ for Swedish R. cirrhosa and 200 µmol photons m⁻² s⁻¹ for Thai R. maritima. The temperatures within the chambers were adjusted with a temperature bath (RC20, Lauda, Lauda-Königshofen, Germany, for the Swedish set-up and MP-10C, Shanghai Bluepard Instruments, Shanghai, China, for the Thai set-up) providing water circulation through jackets surrounding the chambers. The temperature treatments used were 18 (ambient water temperature), 23, 28, and 33 °C in the Swedish set-up (n = 9 per treatment) and 28 (ambient water temperature), 33, 38, and 43 °C in the Thai set-up (n = 10 per treatment). The temperature ranges were chosen based on the temperatures occurring in the plants’ natural habitats, encompassing unusually high temperatures recorded in Swedish and Thai water [53,54] and upper thermal thresholds of temperate and tropical seagrasses reported in previous studies [12,14,16,18–21,23]. The seawater added to the chambers had salinity and pH levels adjusted to ambient field conditions (salinity: ~25/~30 in Sweden/Thailand, respectively; pH: 8.1 for both regions). The water in the chambers was continuously stirred with a magnetic stir bar.

The samples were first incubated in darkness for 15 min. Then the initial maximum photosynthetic efficiency (Fv/Fm) and the PSII potential activity (Fv/F0) were obtained using pulse amplitude-modulated (PAM) fluorometry (Diving-PAM, Walz, Effeltrich, Germany). Values were obtained after dark adaptation where F0 (=minimal fluorescence) and Fm (=maximum fluorescence) were given by the PAM. Fv (=variable fluorescence) was calculated as:

\[ F_v = F_m - F_0 \]
After the dark period, the light (100 and 200 µmol photons m$^{-2}$ s$^{-1}$ for Swedish and Thai set-up, respectively) was turned on and the effective quantum yield ($\Phi_{PSII}$) of photosystem II and excess energy emission through non-photochemical quenching (NPQ) were measured after 20 min in light. The $F_v/F_m$ and $F_v/F_0$ ($F_v/F_m$ final and $F_v/F_0$ final) were re-assessed after dark adaptation.

2.3.2. Photosynthetic Responses to Prolonged Warming Treatment

Subsequent prolonged experiments were run for five consecutive days, where seagrass was either placed in aquaria with control (18 °C and 33 °C for Sweden and Thailand, respectively) or with the heated treatment temperatures (28 °C and 40 °C for Sweden and Thailand, respectively). Two temperature levels were chosen from the first experiment. For the Swedish set-up, the ambient temperature (18 °C) at the sampling site was used as control and 28 °C, corresponding to the SST extreme recorded in the Baltic Sea in 2018 [53], was used as the heated treatment. For the Thai set-up, 33 °C was used as control based on no adverse effect on the $F_v/F_m$ and $F_v/F_0$ observed in the first experiment and 40 °C, corresponding to the temperature extreme recorded in a shallow seagrass habitat (unpublished data), was used as the heated treatment. Note that a trial was initially conducted using a 43 °C heated treatment, which led to an immediate mortality within 24 h. The $F_v/F_m$, $F_v/F_0$, $\Phi_{PSII}$, and NPQ were measured daily at the end of the photoperiod to follow the status of the photosynthetic apparatus of the seagrass over time.

2.4. Statistical Analyses

The statistical analyses were carried out using Statistica version 13. Prior to analysis of variance (ANOVA), homogeneity of variances was tested using Levene’s test [55].

In the first experimental set-up, analysis of covariance (ANCOVA) was used to compare photosynthetic responses in *Ruppia cirrhosa* and *R. maritima* to a series of temperatures. Species was used as the categorical factor and temperature (as 0, 5, 10, and 15 °C increase from ambient temperature) was used as the continuous predictor. The Fisher’s least significant difference (LSD) test was used for post-hoc comparisons to determine species-specific effects on the relationships of each photosynthetic parameter with increasing temperatures. In addition, ANOVAs (repeated ANOVA for $F_v/F_m$ and $F_v/F_0$ and two-way ANOVA for $\Phi_{PSII}$ and NPQ) were used for comparisons of means of the photosynthetic parameters for main factors and for their interactions. The Fisher’s LSD test was used for pairwise multiple comparisons across species, temperature, and time.

ANCOVA was used to assess the differential effects of the prolonged warming treatment on photosynthesis of *R. cirrhosa* and *R. maritima*. Species and treatment were used as categorical factors and treatment duration (day 1–5) was used as the continuous predictor. The Fisher’s LSD test was used for post-hoc comparisons. Repeated-measures ANOVA was used to test for differences of means of the photosynthetic parameters over time. Treatment duration was used as the within group factor and species and temperature treatments were used as the categorical factors. The Fisher’s LSD test was used for pairwise multiple comparisons of means across species, treatments (control and warming treatment), and treatment duration (day after treatment).

3. Results

3.1. Analysis of ITS (ITS1-5.8S-ITS2) and rbcL DNA Sequences

The genes size and accession numbers of the amplified PCR products of ITS sequences containing ITS1-5.8S-ITS2 and *rbcL* are shown in Table 2. The BLASTn analysis of ITS (ITS1-5.8S-ITS2) and *rbcL* sequences confirmed that *Ruppia* sampled in Sweden was close to *Ruppia cirrhosa* with a 100% and 99.85% sequence identity, respectively, while *Ruppia* sampled in Thailand was close to *R. maritima* with a 100% and 99.85% sequence identity, respectively (Table 2). A phylogenetic analysis of the *Ruppia* specimens based on their ITS (ITS1-5.8S-ITS2) and *rbcL* regions was performed and a phylogenetic tree was constructed. The comparison between samples collected from Sweden and Thailand and the
ITS (ITS1-5.8S-ITS2) and rbcL reference sequences retrieved from GenBank ensured that the identification of *Ruppia* samples from Sweden were close to *R. cirrhosa* with the bootstrap supports of 97% (ITS dataset), 50% (rbcL dataset), and 100% (ITS+ rbcL dataset), while *Ruppia* specimens from Thailand were close to *R. maritima* with the bootstrap support of 100% (ITS dataset), 50% (rbcL dataset), and 100% (ITS+ rbcL dataset). *Ruppia megacarpa* was used as an outgoing group (Figure 2).

**Table 2.** The BLASTn of partial ITS and rbcL gene.

| Sample              | Gene | Length (bp) | Accession No. | Homology                  | Query Cover (%) | Total Score | Identity (%) | E-Value |
|---------------------|------|-------------|---------------|---------------------------|-----------------|-------------|--------------|---------|
| *Ruppia cirrhosa*  | ITS  | 711         | MZ474644      | *R. cirrhosa* (AB728749.1) | 100             | 1283        | 100          | 0.0     |
|                     | rbcL | 654         | MZ466378      | *R. cirrhosa* (JN113277.1) | 100             | 1252        | 99.85        | 0.0     |
| *Ruppia maritima*  | ITS  | 710         | MZ453015      | *R. maritima* (AB728734.1) | 100             | 1281        | 100          | 0.0     |
| (Thailand)          | rbcL | 654         | MZ466377      | *R. maritima* (JN113279.1) | 100             | 1252        | 99.85        | 0.0     |

3.2. Temperature Effects on the Photosynthetic Efficiency

3.2.1. Photosynthetic Responses to a Series of Temperatures

*Ruppia* from the two different bioregions were affected by temperature, but to a different extent. A 15 °C increase in temperature (from 18 °C) resulted only in a minor change in the photosynthetic parameters in *Ruppia cirrhosa* (Swedish), but when *R. maritima* (Thai) was exposed to 43 °C (increased from 28 °C) it induced a substantial photoinhibition (Figure 3).

Temperature significantly influenced the maximum quantum yield (F\textsubscript{v}/F\textsubscript{m} initial and final) and the PSII potential activity (F\textsubscript{v}/F\textsubscript{0} initial and final). Moreover, significant effects of species on F\textsubscript{v}/F\textsubscript{m} final and F\textsubscript{v}/F\textsubscript{0} final were encountered (Figure 3A–D, Table 3A). Significant differences between *R. cirrhosa* and *R. maritima* were detected in the functional relationships between temperatures and F\textsubscript{v}/F\textsubscript{m} final, F\textsubscript{v}/F\textsubscript{0} initial, and F\textsubscript{v}/F\textsubscript{0} final (Table 3B). For *R. cirrhosa* (Swedish) (Figure 3A,C), the mean values of F\textsubscript{v}/F\textsubscript{m} and F\textsubscript{v}/F\textsubscript{0} at 28 and 33 °C were slightly lower than in the other temperature treatments (Fisher’s LSD test, p < 0.05, supplementary material). For *R. maritima* (Thai) (Figure 3B,D), exposure to all treatments, except for 33 °C, resulted in a decrease in F\textsubscript{v}/F\textsubscript{m} and F\textsubscript{v}/F\textsubscript{0} from the initial values (Fisher’s LSD test, p < 0.05, supplementary material). The percentage reduction in F\textsubscript{v}/F\textsubscript{m} and F\textsubscript{v}/F\textsubscript{0} were highest in the 43 °C treatment followed by the treatments of 28 and 38 °C, respectively.

Significant interactions of species and temperature on the effective quantum yield of PSII (ΦPSII) and the effects of species on non-photochemical quenching (NPQ) were detected (Figure 3E–H, Table 3A). Furthermore, the functional relationships between temperature and ΦPSII and NPQ were significantly different when comparing *R. cirrhosa* and *R. maritima* (Table 3B). While no difference in the mean values of ΦPSII was detected in *R. cirrhosa* (Swedish) (Figure 3E), a significant change was found in *R. maritima* (Thai) (Figure 3F). An increasing trend in ΦPSII as temperature increases from 28 to 38 °C and a sharp decline at 43 °C were detected (Fisher’s LSD test, p < 0.05, supplementary material). The NPQ of *R. cirrhosa* (Swedish) remained unchanged (Figure 3G), whereas large variation in NPQ across temperatures with a significant decrease at 38 °C was observed in *R. maritima* (Thai) (Figure 3H).
Figure 2. The phylogenetic tree for species identification of *Ruppia* samples from Sweden and Thailand in comparison with other *Ruppia* species using fragments of ITS (ITS1-5.8S-ITS2) and *rbcL*. The evolutionary history was inferred using the maximum likelihood method, Kimura 2-parameter model, and 1000 bootstrap replications; (A) ITS (ITS1-5.8S-ITS2) dataset, (B) *rbcL* dataset, and (C) the combined dataset of ITS (ITS1-5.8S-ITS2) and *rbcL*. 
Figure 3. The effects of experimental temperatures on the photosynthetic parameters, including (A) the maximum quantum yield ($F_{v}/F_{m}$) in *Ruppia cirrhosa* (Swedish), (B) $F_{v}/F_{m}$ in *R. maritima* (Thai), (C) the PSII potential activity ($F_{v}/F_{0}$) in *R. cirrhosa* (Swedish), (D) $F_{v}/F_{0}$ in *R. maritima* (Thai), (E) the effective quantum yield ($\phi_{PSII}$) in *R. cirrhosa* (Swedish), (F) $\phi_{PSII}$ in *R. maritima* (Thai), (G) non-photochemical quenching (NPQ) in *R. cirrhosa* (Swedish), and (H) NPQ in *R. maritima* (Thai). Values are shown as mean ± SE ($n = 9$ for *R. cirrhosa* (Swedish) and $n = 10$ for *R. maritima* (Thai)). Significant differences in mean values across species and treatments are denoted by different letters (Fisher’s LSD test, $p < 0.05$).
Table 3. Summary of A. analysis of covariance (ANCOVA) of four photosynthetic parameters in *Ruppia cirrhosa* (Swedish) and *R. maritima* (Thai) in responses to a series of temperatures and B. the Fisher’s least significant difference (LSD) post-hoc test. Significant values (*p* < 0.05) are shown in bold.

| A. Effect | SS    | Degree of Freedom | MS    | F     | p     |
|-----------|-------|------------------|-------|-------|-------|
| *Fv/Fm initial* |       |                  |       |       |       |
| Species   | 0.004 | 1                | 0.004 | 3.91  | 0.052 |
| Temperature | 0.023 | 1                | 0.023 | 22.65 | <0.001|
| Species x Temperature | 0.002 | 1                | 0.002 | 2.04  | 0.157 |
| Error     | 0.073 | 72               | 0.001 |       |       |
| *Fv/Fm final* |       |                  |       |       |       |
| Species   | 0.011 | 1                | 0.011 | 3.604 | 0.062 |
| Temperature | 0.14  | 1                | 0.14  | 46.6  | <0.001|
| Species x Temperature | 0.074 | 1                | 0.074 | 24.67 | <0.001|
| Error     | 0.217 | 72               | 0.003 |       |       |
| *Fv/Fo initial* |       |                  |       |       |       |
| Species   | 1.563 | 1                | 1.563 | 5.71  | <0.05 |
| Temperature | 4.601 | 1                | 4.601 | 16.805| <0.001|
| Species x Temperature | 0.045 | 1                | 0.045 | 0.165 | 0.685 |
| Error     | 19.714| 72               | 0.274 |       |       |
| *Fv/Fo final* |       |                  |       |       |       |
| Species   | 3.669 | 1                | 3.669 | 12.517| <0.01 |
| Temperature | 8.538 | 1                | 8.538 | 29.128| <0.001|
| Species x Temperature | 1.871 | 1                | 1.871 | 6.382 | <0.05 |
| Error     | 21.104| 72               | 0.293 |       |       |
| ϕPSII     |       |                  |       |       |       |
| Species   | 0.027 | 1                | 0.027 | 2.093 | 0.152 |
| Temperature | 0.027 | 1                | 0.027 | 2.098 | 0.152 |
| Species x Temperature | 0.093 | 1                | 0.093 | 7.151 | <0.01 |
| Error     | 0.937 | 72               | 0.013 |       |       |
| NPQ       |       |                  |       |       |       |
| Species   | 8.142 | 1                | 8.142 | 9.616 | <0.01 |
| Temperature | 0.109 | 1                | 0.109 | 0.129 | 0.72  |
| Species x Temperature | 0.002 | 1                | 0.002 | 0.002 | 0.962 |
| Error     | 60.965| 72               | 0.847 |       |       |

| B. Effect | Mean Square Error | Degree of Freedom | p    |
|-----------|------------------|------------------|------|
| *Fv/Fm final* | 0.003     | 72               | <0.001|
| *Fv/Fo initial* | 0.274     | 72               | <0.001|
| *Fv/Fo final* | 0.293     | 72               | <0.001|
| ϕPSII     | 0.013     | 72               | <0.001|
| NPQ       | 0.847     | 72               | <0.001|

3.2.2. Photosynthetic Responses to Prolonged Warming Treatments

Two levels of temperature were chosen for a longer exposure experiment (18 and 28 °C for *Ruppia cirrhosa* (Swedish) and 33 and 40 °C for *R. maritima* (Thai)). Time course responses to warming displayed in Figure 4 show a slight photoinhibition in *R. cirrhosa* (Swedish) and a deleterious effect in *R. maritima* (Thai).

Analysis of covariance (ANCOVA, Table 4A) showed significant effects of species and treatments and interactions between these factors for time course responses of all photosynthetic parameters (Figure 4). The Fisher’s LSD test showed that the regression slopes of the photosynthetic parameters (*Fv/Fm*, *Fv/Fo*, ϕPSII, and NPQ) over time in *R. maritima* in the warming treatment differed significantly from those derived from the other species and treatments (Table 4B), while no significant difference was found among *R. cirrhosa* (control), *R. cirrhosa* (warming treatment), and *R. maritima* (control).
Figure 4. Time course of the warming effects on the photosynthetic parameters, including (A) the maximum quantum yield (Fv/Fm) in *Ruppia cirrhosa* (Swedish), (B) Fv/Fm in *R. maritima* (Thai), (C) the PSII potential activity (Fv/F0) in *R. cirrhosa* (Swedish), (D) Fv/F0 in *R. maritima* (Thai), (E) the effective quantum yield (φPSII) in *R. cirrhosa* (Swedish), (F) φPSII in *R. maritima* (Thai), (G) non-photochemical quenching (NPQ) in *R. cirrhosa* (Swedish), and (H) NPQ in *R. maritima* (Thai). Values are shown as mean ± SE (n = 6 for *Ruppia cirrhosa* (Swedish) and n = 6 for *Ruppia maritima* (Thai)). Significant differences in mean values from controls within the same day of measurements are denoted by * (Fisher’s LSD test, p < 0.05).
Table 4. Summary of A. analysis of covariance (ANCOVA) of four photosynthetic parameters in *Ruppia cirrhosa* (Swedish) and *R. maritima* (Thai) in responses to prolonged warming treatments and B. the Fisher’s least significant difference (LSD) post-hoc test comparing *R. maritima* in warming treatment to *R. maritima* (control), *R. cirrhosa* (control), and *R. cirrhosa* (warming treatment). Significant values (*p* < 0.05) are shown in bold.

| A. Effect | SS     | Degree of Freedom | MS   | F       | *p*     |
|-----------|--------|-------------------|------|---------|---------|
| *F*<sub>v</sub>/F<sub>m</sub> |        |                   |      |         |         |
| Species   | 1.488  | 1                 | 1.488| 82.668  | <0.001  |
| Treatment | 0.968  | 1                 | 0.968| 53.75   | <0.001  |
| Species x Treatment | 0.734 | 1                 | 0.734| 40.794  | <0.001  |
| Error     | 2.07   | 116               | 0.018|         |         |
| *F*<sub>v</sub>/F<sub>0</sub> |        |                   |      |         |         |
| Species   | 57.469 | 1                 | 57.469| 107.723 | <0.001  |
| Treatment | 27.813 | 1                 | 27.813| 52.135  | <0.001  |
| Species x Treatment | 11.564 | 1                 | 11.564| 21.677  | <0.001  |
| Error     | 61.885 | 116               | 0.533|         |         |
| ϕ<sub>PSII</sub> |        |                   |      |         |         |
| Species   | 0.724  | 1                 | 0.724| 54.05   | <0.001  |
| Treatment | 0.871  | 1                 | 0.871| 65.054  | <0.001  |
| Species x Treatment | 0.935 | 1                 | 0.935| 69.805  | <0.013  |
| Error     | 1.553  | 116               | 0.013|         |         |
| NPQ       |        |                   |      |         |         |
| Species   | 11.932 | 1                 | 11.932| 23.823  | <0.001  |
| Treatment | 2.78   | 1                 | 2.78 | 5.551   | <0.05   |
| Species x Treatment | 2.946 | 1                 | 2.946| 5.882   | <0.05   |
| Error     | 58.097 | 116               | 0.501|         |         |

| B. Effect | Mean Square | Degree of Freedom | *p*     | *R. maritima* (Control) | *p*     | *R. cirrhosa* (Control) | *p*     |
|-----------|-------------|-------------------|---------|-------------------------|---------|-------------------------|---------|
| *F*<sub>v</sub>/F<sub>m</sub> | 0.018       | 116               | <0.001  | <0.001                  | <0.001  |
| *F*<sub>v</sub>/F<sub>0</sub> | 0.533       | 116               | <0.001  | <0.001                  | <0.001  |
| ϕ<sub>PSII</sub> | 0.013       | 116               | <0.001  | <0.001                  | <0.001  |
| NPQ       | 0.501       | 116               | <0.01   | <0.001                  | <0.001  |

For *Ruppia cirrhosa* (Swedish), the three parameters measured in controls remained stable throughout the experiment. A significant difference in the maximum quantum yield of PSII (*F*<sub>v</sub>/F<sub>m</sub>) (Figure 4A) between controls and the warming treatment was detected on day 5 (repeated-measures ANOVA and Fisher’s LSD test, *p* < 0.05, supplementary material), whereas a significant difference in the PSII potential activity (*F*<sub>v</sub>/F<sub>0</sub>) (Figure 4C) between controls and warming treatment was detected from day 3 onwards (repeated-measures ANOVA and Fisher’s LSD test, *p* < 0.05, supplementary material). No significant difference in mean values of the effective quantum yield (ϕ<sub>PSII</sub>) (Figure 4E) and non-photochemical quenching (NPQ) (Figure 4H) between controls and the warming treatment was detected.

For *Ruppia maritima* (Thai), the *F*<sub>v</sub>/F<sub>m</sub> measured in controls remained stable throughout the experiment, whereas a significant decline in *F*<sub>v</sub>/F<sub>m</sub> in the warming treatment, thus causing a significant difference from controls, was detected from day 2 onwards (repeated-measures ANOVA and Fisher’s LSD test, *p* < 0.05, supplementary material). Further, a decline was observed on day 4 and day 5, reaching almost zero. Significant variations in *F*<sub>v</sub>/F<sub>0</sub> (Figure 4D) were observed in the controls (repeated-measures ANOVA and Fisher’s LSD test, *p* < 0.05, supplementary material); however, the *F*<sub>v</sub>/F<sub>0</sub> measured in the warming treatment on day 2 and onwards was significantly lower than the controls (repeated-measures ANOVA and Fisher’s LSD test, *p* < 0.05, supplementary material). The ϕ<sub>PSII</sub> (Figure 4F) measured in controls remained stable throughout the experiment, whereas ϕ<sub>PSII</sub> measured in the warming treatment displayed a steady decline (repeated-measures ANOVA and Fisher’s LSD test, *p* < 0.05, Supplementary Material). No significant variation in NPQ was detected in controls (Figure 4H). An increase in NPQ was observed in the warming treatment on day 2 and day 3 followed by a notable decrease. This
resulted in a significant difference in NPQ between controls and the warming treatment on days 2 and 3 (Figure 4H, repeated-measures ANOVA and Fisher’s LSD test, \( p < 0.05 \), supplementary material). It is worth noting that mortality was observed at the end of the experiment, which corresponded to the chlorophyll fluorescence parameters that were found approaching zero.

4. Discussion

The results demonstrate distinguished photosynthetic responses to warming above ambient levels between temperate \textit{Ruppia cirrhosa} and tropical \textit{R. maritima}, most likely depending on how close to their thermal tipping point the plants are growing in their natural habitat. Temperate \textit{R. cirrhosa} exhibited high tolerance to increasing temperature, indicating that in this region \textit{R. cirrhosa} might not suffer from future predicted warming scenarios. On the contrary, tropical \textit{R. maritima} showed severe sensitivity to increased temperatures, suggesting that in tropical waters the \textit{Ruppia} productivity and distribution may be reduced if seawater temperatures continue to rise as predicted.

Analysis of ITS (ITS1-5.8S-ITS2) and \textit{rbcL} DNA sequences indicated that samples of \textit{Ruppia} from Sweden was close to \textit{Ruppia cirrhosa}, while samples from Thailand was close to \textit{Ruppia maritima}. Nevertheless, \textit{Ruppia} sampled from the two different bioregions shared highly similar sequences of ITS (ITS1-5.8S-ITS2) and \textit{rbcL} (Figure S2 and S3). Phylogenetic placement of species within the family \textit{Ruppiaceae} is a debated and difficult task because of their reduced morphology, high intraspecific variability, and hybridization [45,56]. While \textit{R. maritima} and \textit{R. cirrhosa} were identified as separate species [45,56–61], three to four species and one cosmopolitan species complex (containing several lineages, including \textit{R. maritima} and \textit{R. cirrhosa}) have been proposed based on molecular evidence showing hybridization and a variety in polyploidy [57–61]. In addition, typification issues were raised by den Hartog and Triest [56] and \textit{Ruppia spiralis} L. ex Dumortier was proposed as a more correct name for \textit{R. cirrhosa}. Our study, however, focused on comparing thermal sensitivity of \textit{Ruppia} from contrasting temperature regimes, addressing the effects of warming at the genus level.

\textit{Ruppia} from the two different bioregions had clear differences in their photosynthetic response to increased temperature. Overall, tropical \textit{R. maritima} from Thailand was more sensitive to the tested temperatures than the temperate Swedish \textit{Ruppia cirrhosa}, whether looking at direct effects at four different temperatures or over a five-day period of heat exposure in comparison to control levels. It is considered that marine tropical species are more sensitive to increased temperatures than temperate species, as tropical species are in an evolutionary point of view adjusted to environments that are more constant [39–41]. Moreover, the ambient temperatures in tropical habitats are assumed to be closer to the upper thermal threshold of the organisms [23,39–41]. Thus, warming events in tropical areas may push the organisms beyond their operating and survival limits as demonstrated in our results.

A few data are available on thermal biology of \textit{R. cirrhosa} from Sweden and the surrounding areas, while populations from the Mediterranean have been extensively investigated [17,46]. In the Mediterranean, \textit{R. cirrhosa} has been shown to survive temperatures up to 38 °C [17,46], however, with a clear decline in photosynthetic performance at 36 °C [17] and thermal optima of between 20 and 30 °C [62]. As the Swedish \textit{R. cirrhosa} was quite unaffected by temperature increase, it seems that the individuals can sustain their photosynthesis within the general temperature acceptance of the species reported in the Mediterranean populations and above the temperature range occurring in their habitats [17,46]. It needs to be emphasized that some of the tested temperatures (28–33 °C) are not commonly encountered in Swedish water. During summer months, the highest temperature recorded in shallow bays at the Swedish west coast, environments often inhabited with \textit{Ruppia}, was 22–23 °C [63,64]. Swedish SST generally does not exceed 24 °C; however, in extreme years, such as 2018, a SST of 28 °C was measured in the Baltic Sea [53]. In addition, predicted future scenarios indicate that the temperature in the Västra Götaland
County on the Swedish west coast is expected to increase by almost 3 °C by the end of the century according to RCP4.5 and close to 5 °C according to RCP8.5. RCP8.5 also shows an increase in the number of very warm days, with an annual average of 18 consecutive days with daily average temperatures above 20 °C at the end of the century [65]. Even so, the predicted future temperature scenarios may not be as detrimental for the productivity and performance of the Swedish R. cirrhosa as for the Thai species.

Direct or indirect damage of the photosynthetic machinery at temperatures above the thermal tolerance of a plant may be related to protein degradation, hampering of protein synthesis, or decreased membrane stability [24]. Lowered Fv/Fm and Fm and increased F0 during heat stress have previously been attributed to loss of membrane integrity [66], and hence it may be a plausible explanation to the lowered Fv/Fm and Fv/F0 values encountered in especially the Thai R. maritima of this study. Nevertheless, even though the Fv/Fm was lowered with higher temperature, the effective quantum yield (φPSII) was highest at 38 °C, indicating that the light-driven photochemistry is still working at this temperature. However, the remaining φPSII may be sustained by potential alternative electron transports, such as photorespiration and the Mehler reaction [67]. Fv/Fm is a conventional methodological approach when assessing maximum quantum yield of PSII [68–70]. However, the less commonly used variable chlorophyll fluorescence ratio Fv/F0 (indicating PSI potential activity) could be considered a more sensitive parameter detecting stress upon the photosynthetic apparatus at an earlier stage and with a stronger signal [19,71,72]. This was specifically clear in the response of the Swedish R. cirrhosa in prolonged temperature exposure, and it might be discussed that this ratio is preferable in future measurements of PSII activity, in order to capture plant stress on a more detailed level. The φPSII, indicating the working capacity of the photosynthetic apparatus, did not reveal any great differences in the Swedish R. cirrhosa, even though the optimum temperature seemed to be at 23 °C. The optimum temperature of φPSII at 38 °C for the Thai R. maritima was followed by a great decline at 43 °C. This may indicate that at high temperature, captured energy is not used for photochemistry, but dissipated through non-photochemical quenching. There were no clear signs of NPQ contribution upon immediate temperature stress; however, after prolonged exposure a brief increase in NPQ was detected in the Thai R. maritima suggesting photoprotective efforts of the plants. In other seagrass species, e.g., Posidonia oceanica and Zostera muelleri, an increase in NPQ with temperature has been attributed to the activation of the xanthophyll cycle as a protective measure to discard excess energy [73,74]. However, when reaching a thermal threshold, NPQ was lowered [74], suggesting that also NPQ in seagrass is sensitive to extreme temperatures. As NPQ formation requires a transmembrane proton gradient, compromised integrity of biological membranes caused by heat stress may contribute to a decline in NPQ at high temperature [75]. Heating events may thus affect seagrass physiology in several negative ways, not just impaired photosynthesis but also a decrease in protective functions and direct biomass loss [20,23]. Moreover, with increased temperature, there might be an overall lowering of primary productivity due to photorespiration. This is particularly important in conditions of high oxygen and high temperature [64], often encountered in shallow vegetated areas [76]. The Thai R. maritima seems more resistant to high temperature than the specimens from the Mediterranean and Tampa Bay, Florida [17,46]. It remains to be elucidated whether local adaptation to the tropical climate may have shifted their thermal threshold. Nevertheless, the thermostolerance range of the Thai R. maritima might not allow this species to cope with extreme warming events in the tropical latitudes. While the prediction of future increase in SST in Thailand is not yet available, exceptionally warm temperatures exceeding 40 °C have been recorded in tropical intertidal seagrass meadows [20,23]. This implies that the Thai R. maritima may suffer as temperature rises, especially when more frequent extreme weather events and larger numbers of warm days are expected as a result of a changing climate [75].

Photosynthesis of the Swedish R. cirrhosa seemed intact with direct and prolonged exposure to increased temperature. Hence, a predicted future temperature increase may
not affect Swedish \textit{R. cirrhosa} in the same negative way as \textit{Z. marina}, a seagrass species that in the same geographic area showed photoinhibition at lower temperatures [19]. Although community dynamics resulted from complex interactions among species and environmental drivers, our short-term data may provide supporting evidence for some ecological observations reported earlier. The authors of previous field investigations have discussed potential replacement of more slow growing climax species such as \textit{Zostera marina} in favor for the fast-growing colonizing opportunist \textit{R. maritima} in high temperature scenarios [11,77,78]. In North American waters, shifts from \textit{Z. marina} to \textit{R. maritima}-dominated systems have been reported as \textit{R. maritima} colonizes areas that are opened up by \textit{Z. marina} die-backs, most likely due to the higher tolerance of \textit{Ruppia} to temperature and salinity change [11,77,78]. From an ecological point of view, a dominance shift from \textit{Zostera} to \textit{Ruppia} had no effects on canopy invertebrate assemblages, suggesting that the two seagrasses could harbor similar function for marine fauna. However, \textit{Ruppia} had a lower sediment retention due to coarsening of the bottom substrate [79]. Hence, some ecosystem services provided by one seagrass species may not be lost due to a shift in dominance, while some may vanish. Moreover, in experiments simulating nitrate enrichment as a form of eutrophication, shoot production of \textit{R. maritima} was increased by more than 300\%, while \textit{Z. marina} showed a reduction in number of shoots [80]. \textit{Ruppia} has different growth cycles than other seagrasses, where they start developing shoots in winter months when most other species are dormant. In addition, their die-back in fall is earlier than most other species. This different timing might pose a colonization advantage [43]. These different examples highlight the broad adaptive capability of this genus over many other seagrass species to environmental change, something that according to our photosynthetic response to temperature results could be especially important in Swedish waters, where \textit{Ruppia} seems to have higher tolerance threshold than the co-occurring species [12,19].

In tropical seas, including Thai waters, other seagrass species tolerate similar and higher temperatures [15,19,20] than the \textit{R. maritima} of this study, indicating that \textit{R. maritima} may be outcompeted in tropical environments. However, the life history of opportunistic traits combined with their environmental sturdiness may allow them to recover from short-term heat stress. Furthermore, \textit{Ruppia} is able to withstand salinities from 0 to 70, whereas other seagrasses are restricted to 5–45 [81]. Moreover, there is accumulating evidence suggesting that thermal sensitivity, measured as short-term physiological responses, is not necessarily translated to the acclimation capacity in various organisms [82–84]. In a longer term, organisms may be able to acclimate to a warming environment [82–84]. Additionally, acclimation capacity may differ significantly among species and populations. Long-term studies exploring species’ acclimation capacity as well as complex biotic interactions and multiple environmental drivers are necessary to improve the prediction of the fate of \textit{Ruppia} populations in the face of climate change.

5. Conclusions

This study clearly demonstrates that \textit{Ruppia} from two different bioregions have very different responses in photosynthetic capacity to predicted future water temperature increases. The Swedish temperate \textit{R. cirrhosa} was able to maintain photosynthetic efficiency at temperatures way above their ambient conditions, indicating that they will likely withstand a future increase of ocean temperature and warming events. As the other dominant seagrass in this geographic area, \textit{Zostera marina}, is less tolerant to temperature increase, climate change may lead to a shift towards a \textit{Ruppia}-dominant system in Swedish waters. On the contrary, Thai \textit{R. maritima} were negatively affected by an increase in temperature, directly and over time, indicating that individuals are close to their thermal threshold and more sensitive to warming scenarios. Hence, in Thai waters, climate change effects may more likely trigger a shift in the coastal community towards a more negative outcome for \textit{Ruppia} abundance.
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su13169433/s1, Figure S1: Experimental design and timeline of the measurements. Figure S2: The multiple sequence alignment result of ITS gene sequences of the *Ruppia cirrhosa* from Sweden (*Ruppia_sp_SW*) and *R. maritima* from Thailand (*Ruppia_sp_TH*) with the reference strains; *R. maritima* (Accession No. AB728734.1) and *R. cirrhosa* (Accession No. AB728749.1) using MUSCLE (MUltiple Sequence Comparison by Log Expectation). Figure S3: The multiple sequence alignment result of *rbcL* gene sequences of the *R. cirrhosa* from Sweden (*Ruppia_sp_SW*) and *R. maritima* from Thailand (*Ruppia_sp_TH*) with the reference strains; *R. maritima* (Accession No. JN113279.1) and *R. cirrhosa* (Accession No. JN113277.1) using MUSCLE (Multiple Sequence Comparison by Log Expectation).

Author Contributions: Conceptualization, L.M.R., A.N.-o., M.B. and P.B.; Data curation, L.M.R., A.N.-o., T.W. and P.B.; Formal analysis, L.M.R., A.N.-o. and P.B.; Funding acquisition, L.M.R. and P.B.; Investigation, L.M.R., A.N.-o., T.W., M.B., M.G. and P.B.; Methodology, L.M.R., A.N.-o., M.B. and P.B.; Project administration, P.B.; Resources, L.M.R., A.N.-o., M.B. and P.B.; Software, A.N.-o. and P.B.; Supervision, L.M.R., A.N.-o. and P.B.; Visualization, A.N.-o. and P.B.; Writing—Original draft, L.M.R., A.N.-o., M.B., M.G. and P.B.; Writing—Review and editing, L.M.R., A.N.-o., M.B., M.G. and P.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by National Science, Research and Innovation Fund (NSRF) and Prince of Songkla University (Grant Number: ENV6405083M) and KVA fund for scientific renewal and internationalization at the Sven Lovén Centre 2019 for P.B. and mobility grant from the Swedish research council FORMAS, grant number 2017-00363 for L.M.R. The APC was funded by National Science, Research and Innovation Fund (NSRF) and Prince of Songkla University (Grant Number: ENV6405083M).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequence data supporting the findings of this study are available in GenBank with the following accession codes, AB728749.1 (*R. cirrhosa ITS* gene), MN958127.1 (*R. drepanensis* ITS gene), AB728734.1 (*R. maritima* ITS gene), JQ034337.1 (*R. megacarpa* ITS gene), JN113279.1 (*R. maritima rbcL* gene), NC051974.1 (*R. brevipedunculata rbcL* gene), JN113277.1 (*R. cirrhosa rbcL* gene), MN233650.1 (*R. sinensis rbcL* gene), and AB507891.2 (*R. megacarpa rbcL* gene), MZ453015 (*R. maritima ITS* gene (Thailand)), MZ466377 (*R. maritima rbcL* gene (Thailand)), MZ474644 (*R. cirrhosa ITS* gene (Sweden)), and MZ466378 (*R. cirrhosa rbcL* gene (Sweden)).

Acknowledgments: We wish to thank Somsak Buatip for his help with sample collection from Pattani Province.

Conflicts of Interest: The authors declare no conflict of interest.

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