

Original Article

Leaf gas films, underwater photosynthesis and plant species distributions in a flood gradient

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ABSTRACT

Traits for survival during flooding of terrestrial plants include stimulation or inhibition of shoot elongation, aerenchyma formation and efficient gas exchange. Leaf gas films form on superhydrophobic cuticles during submergence and enhance underwater gas exchange. The main hypothesis tested was that the presence of leaf gas films influences the distribution of plant species along a natural flood gradient. We conducted laboratory experiments and field observations on species distributed along a natural flood gradient. We measured presence or absence of leaf gas films and specific leaf area of 95 species. We also measured, gas film retention time during submergence and underwater net photosynthesis and dark respiration of 25 target species. The presence of a leaf gas film was inversely correlated to flood frequency and duration and reached a maximum value of 80% of the species in the rarely flooded locations. This relationship was primarily driven by grasses that all, independently of their field location along the flood gradient, possess gas films when submerged. Although the present study and earlier experiments have shown that leaf gas films enhance gas exchange of submerged plants, the ability of species to form leaf gas films did not show the hypothesized relationship with species composition along the flood gradient.

Key-words: air films; flooding tolerance; leaf respiration; leaf traits; specific leaf area; submergence tolerance; superhydrophobicity; underwater gas exchange.

INTRODUCTION

Flooding of terrestrial plants has a large impact on species zonation because of survival variation between species and subsequent succession following floods (Turner *et al.* 1998). Flood duration, water depth and temperature act as survival filters during flooding (Keddy 1992), and subsequent survival of plants in flood-prone areas is determined by specific functional traits coping with various aspects of the flooding

stress. These include, capacity for (1) shoot elongation and hyponasty to restore air contact (Voeselek *et al.* 2003b; Voeselek *et al.* 2004); (2) metabolic conservation of carbohydrates (Voeselek *et al.* 2006; Bailey-Serres and Voeselek 2008); (3) production of carbohydrates in underwater photosynthesis (Pedersen, Rich, and Colmer 2009; Colmer, Winkel, and Pedersen 2011); (4) efficient use of carbohydrates by enhanced O₂ exchange with the floodwater enabling oxidative phosphorylation during respiration (Pedersen *et al.* 2009; Winkel *et al.* 2014); and (5) production of adventitious roots with a high porosity when waterlogged for weeks (Visser *et al.* 2000; Colmer 2003) and high porosity in shoot tissues when submerged (Herzog and Pedersen 2014). Moreover, seed bank characteristics and timing of germination can also be of importance for the distribution of species in river floodplains (Voeselek and Blom 1992; Voeselek, De Graaf, and Blom 1992; Van der Sman, Joosten, and Blom 1993; Colmer and Voeselek 2009). While traits such as root porosity and control of shoot elongation are relatively well studied (Colmer and Voeselek 2009; Voeselek and Bailey-Serres 2015), traits enhancing gas exchange between the shoot and the floodwater have only sporadically been considered as drivers for patterns of species distribution in floodplains (e.g. Mommer *et al.* 2006). Hence, the present study focuses on the possible prevalence of high specific leaf area (SLA) and leaf gas films for species in flood-prone areas, because thin leaves (Madsen and Sand-Jensen 1991) and/or gas films (Colmer *et al.* 2011; Verboven *et al.* 2014) facilitate O₂ and CO₂ exchange with floodwaters.

Resistance to gas exchange by plants with the floodwater is high because of the 10 000 times slower diffusion of gases in water compared with in air (Armstrong 1979). The total resistance to gas exchange between leaves and water can be divided into two major components: (1) the diffusive boundary layer (DBL) and (2) resistance of plant tissues (cuticle, stomata and internal tissues) (e.g. Verboven *et al.* (2014). DBL resistance is determined by the thickness of this viscous layer that is influenced by flow velocity, turbulence of the overlying water (Vogel 2006) and leaf morphology (Colmer *et al.* 2011). While leaves of terrestrial species lack features to reduce

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DBL thickness when submerged, aquatic plants have evolved, for example, filamentous leaves and leaves with undulating edges resulting in greatly reduced DBL (Koch 1994; Colmer *et al.* 2011). Tissue resistance is reduced in thin leaves, that is, shorter diffusion path (Madsen and Sand-Jensen 1991), in both terrestrial and aquatic species. Aquatic plants have evolved more advanced anatomical features to minimize internal resistances to gas movements with the most effective being extremely thin leaves, sometimes only two cell layers (Tomlinson 1982) with strongly reduced thickness or transport resistance of the cuticle (Frost-Christensen, Jørgensen, and Floto 2003; Frost-Christensen and Floto 2007), chloroplasts in the epidermis and porous tissues (Arber 1920; Sculthorpe 1967). A reduced cuticle would not be a suitable trait for terrestrial leaves because of the increased risk of desiccation. We hypothesize that in a natural flood gradient, species with thin leaves (high SLA) dominate in the 'often flooded' end of the gradient where complete inundation is more frequent than in the less frequently flooded or drier end (Violle *et al.* 2011; Baastrop-Spohr *et al.* 2015).

In addition to having thinner leaves, some terrestrial plant species can further decrease the total resistance to gas exchange with the floodwater by retention of gas films on the surface of leaves when submerged. Leaf gas films are formed on the surfaces of superhydrophobic leaf cuticles when these are submerged in water (Colmer and Pedersen 2008). Leaf superhydrophobicity was first established as a self-cleansing mechanism (Barthlott and Neinhuis 1997; Neinhuis and Barthlott 1997), and it has since also been shown to enhance underwater photosynthesis, dark respiration and internal aeration of completely submerged plants both in laboratory experiments (Colmer and Pedersen 2008; Pedersen *et al.* 2009) and in field situations (Winkel, Colmer, and Pedersen 2011; Winkel *et al.* 2013). Leaf gas films greatly increase the gas–water interface, and on average, the total resistance to gas exchange between leaf and the surrounding water is reduced, by about fivefold, compared with a situation where the gas film had been experimentally removed (Colmer and Pedersen 2008; Pedersen *et al.* 2009; Verboven *et al.* 2014). Formation of leaf gas films has been demonstrated to occur in eight species of natural wetland plants (Raskin and Kende 1983; Colmer and Pedersen 2008; Malik, English, and Colmer 2009; Teakle, Colmer, and Pedersen 2014; Konnerup *et al.* 2015) including the invasive marsh plant *Spartina anglica* (Winkel *et al.* 2011; Lauridsen *et al.* 2014) and also on the wetland crop rice, *Oryza sativa* (Raskin and Kende 1983; Pedersen *et al.* 2009). Interestingly, a gas film is also observable on leaves of many dryland crops if submerged, such as wheat, barley and oats (Raskin and Kende 1983), but gas film retention time in these situations has not been evaluated. Colmer & Pedersen (2008) found that the leaf gas films on some of the species in their study lasted at least for 2 weeks (e.g. *Phragmites australis*), whereas it only lasted 3 d for *Melilotus siculus* (Teakle *et al.* 2014). In a study of four rice genotypes, gas film retention varied from 4 to 6 d of submergence (Winkel *et al.* 2014). Based on the benefits of leaf gas films during complete submergence, the retention time could be a key factor influencing the duration of plant survival under water. There is little information on how common the

gas film feature is amongst a variety of species, and therefore, one of the aims of the present study was to screen a great number of species within a natural flood gradient for the presence or absence of leaf gas films.

We tested two hypotheses related to leaf traits (SLA and leaf gas film presence) and species occurrences along a flood gradient: (1) having thin leaves (high SLA values) is a leaf trait of plant species inhabiting the 'often flooded' end of the flood gradient, that is, experiencing frequent floods; and (2) having leaf gas films is a trait that favours survival in zones with floods that may be frequent but of relatively short duration. We tested these hypotheses using field investigations (SLA and presence-absence of leaf gas films of 95 species), laboratory experiments (underwater photosynthesis and respiration and gas film retention time of 25 species representing each end of the flood gradient) and a comprehensive database on flood occurrence (frequency and duration) and species presence-absence in a maximum of 92 relevées (plots of vegetation, analysed as a sample of a wider area) as published by Voeselek *et al.* (2004).

MATERIALS AND METHODS

This study relied on data from a database already available on species composition and flood occurrence (frequency and duration; Fig. 1) for the floodplains of the river Waal in the Netherlands dating from 1972 to 1991 (Voeselek *et al.* 2004) and on a field survey and observations of leaf gas film presence-absence (conducted in early summer 2011) for 95 species. The purpose of the survey in 2011 was to establish which plant species possessed leaf gas films and to conduct SLA measurements and not to determine the species composition of the relevées. These new data on leaf traits were then analysed along with species distributions as related to flood occurrences from the database in the study of Voeselek *et al.* (2004). This field survey was followed by laboratory studies on underwater gas exchange and gas film retention time for 25 selected species from the field, 14 of which had the ability to form leaf gas films. The database included abundance of species, but in order to disregard seasonal variation and other factors that influence the abundance at a given time, apart from the flooding events, we only used presence-absence of species in our analyses.

Screening of gas film presence

Leaf gas films are thin layers of gas (50–100 μm thick) retained on the surface of superhydrophobic submerged leaves. Leaf surface properties such as spatial structures (e.g. trenches and grooves), papillae, trichomes and wax platelets in the cuticle can all contribute to the hydrophobicity of a leaf (Koch, Bhushan, and Barthlott 2009; Barthlott *et al.* 2010) and enable it to retain a leaf gas film when submerged. Ninety-five species were tested for presence-absence of leaf gas films (Table 1) immediately after immersion in water when sampled in the field at various locations on the Waal floodplains in the Netherlands. Plants were identified to species, and the shoots, top of shoots or first fully expanded leaf (depending on size of the plant relative to the beaker used for testing) was submerged in DI

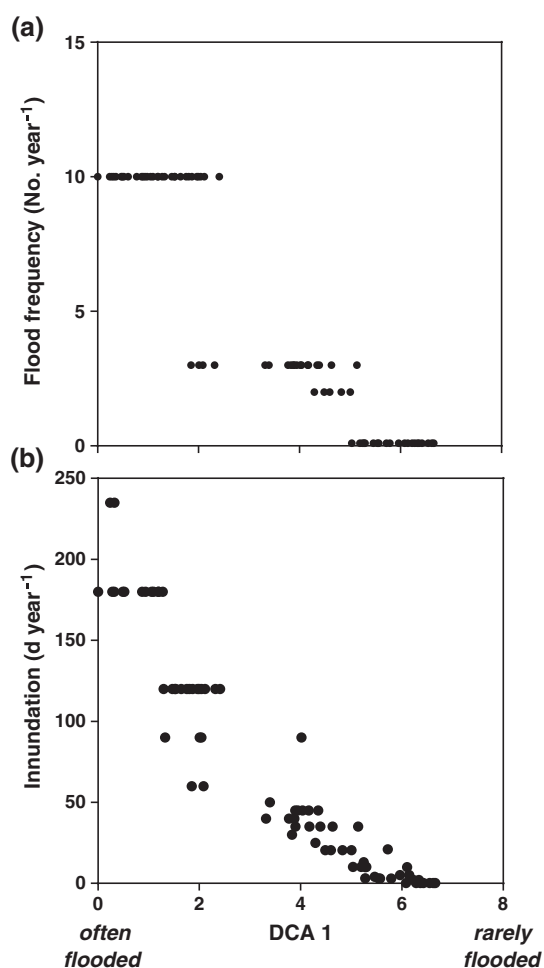


Figure 1. Relation between measures of flooding and DCA 1. Spearman's rank correlation analyses between DCA axis 1 site scores and environmental variables were used to test the primary gradients at the study site. The strongest correlation between environmental variables and the primary gradient in species data (DCA 1) were 'flood frequency' (per year, $r = -0.918$, $P < 0.0001$) and 'inundation' (i.e. days of flooding per year) ($r = -0.968$, $P < 0.0001$) (a and b). Relevées with low DCA 1 values experience up to 10 floods per year with inundation duration up to 235 d per year, or on average, each flood lasts 9 to 23 d. Relevées with high DCA 1 values can experience as little as one flood in a 10 year period which lasts up to 2 d.

water (De-ionized water) in a glass beaker. If gas films were present, they were clearly visible as a silvery sheen on leaf surfaces when held under water at an angle to incident sunlight. Gas films were scored as absent, present on the adaxial side, present on the abaxial side of the leaves, present on both sides of the leaves or present on the stem (for *Equisetum arvense*) or leaf sheath (for grasses).

Specific leaf area

Leaves (for *E. arvense* stems were used) of species tested for presence-absence of leaf gas films were brought back to the laboratory in Zip-lock® bags. The projected area (i.e. one-sided) of the youngest fully expanded leaf was measured using an area metre (CI-202, CID Bio-Science Inc., Camas, WA, USA), and samples were dried until constant weight at 65 °C

Table 1. SLA (first fully expanded leaf, $n = 1$) and presence-absence (+ or –, respectively) of gas films on the stem (or sheath in case of grasses), the adaxial leaf side and the abaxial leaf side of 95 plant species found in the Waal river area

Species	SLA ($\text{m}^2 \text{kg}^{-1} \text{DM}$)	Stem	Gas film (presence-absence)	
			Leaf adaxial side	Leaf abaxial side
<i>Achillea millefolium</i>	15.1	+	-	+
<i>Agrostis stolonifera</i>	60.8	+	+	+
<i>Alisma lanceolatum</i>	33.3	-	-	-
<i>Alopecurus pratensis</i>	19.3	+	+	+
<i>Anthriscus sylvestris</i>	26.7	-	-	-
<i>Aristolochia clematidis</i>	26.7	+	+	+
<i>Arrhenatherum elatius</i>	14.2	-	+	+
<i>Artemisia vulgaris</i>	23.8	+	-	+
<i>Bidens tripartita</i>	34.7	-	-	-
<i>Bellis perenne</i>	32.3	-	-	-
<i>Brassica napus</i>	24.9	+	+	+
<i>Bromus hordeaceus</i>	38.7	-	+	+
<i>Calamagrostis epigeios</i>	14.0	-	+	+
<i>Capsella bursa-pastoris</i>	16.6	-	-	-
<i>Carex arenaria</i>	n.d.	-	-	-
<i>Carex hirta</i>	24.8	-	-	-
<i>Centaurium pratense</i>	20.8	+	+	+
<i>Cerastium arvense</i>	16.2	-	-	-
<i>Cerastium fontanum</i>	22.4	-	-	-
<i>Chenopodium glaucum</i>	n.d.	n.d.	+	+
<i>Chenopodium rubrum</i>	n.d.	n.d.	-	-
<i>Chrysanthemum leucanthemum</i>	15.8	-	-	-
<i>Cirsium arvense</i>	19.3	-	-	-
<i>Crataegus monogyna</i>	18.3	-	-	-
<i>Crepis biennis</i>	34.3	-	-	-
<i>Cynodon dactylon</i>	23.2	-	+	+
<i>Dactylis glomerata</i>	30.1	+	+	+
<i>Daucus carota</i>	24.1	-	-	-
<i>Eleocharis acicularis</i>	n.d.	n.d.	-	-
<i>Eleocharis palustris</i>	10.2	-	-	-
<i>Elymus repens</i>	22.3	n.d.	+	+

(Continues)

Table 1. (Continued)

Species	SLA (m ² kg ⁻¹ DM)	Stem	Gas film (presence-absence)	
			Leaf adaxial side	Leaf abaxial side
<i>Equisetum arvense</i>	12.3	+	+	+
<i>Equisetum hyemale</i>	4.7	+	-	-
<i>Eryngium campestre</i>	9.7	+	+	+
<i>Euphorbia cyparissias</i>	24.0	-	+	+
<i>Festuca arundinacea</i>	20.6	-	+	-
<i>Festuca rubra</i>	12.2	-	+	-
<i>Galium molle</i>	22.4	-	-	-
<i>Geranium pusillum</i>	n.d.	-	-	-
<i>Glechoma hederacea</i>	25.6	-	-	-
<i>Glyceria maxima</i>	26.6	-	-	+
<i>Heracleum sphondylium</i>	28.8	-	-	+
<i>Holcus lanatus</i>	12.8	+	+	+
<i>Hottonia palustris</i>	32.6	-	-	-
<i>Juncus compressus</i>	13.0	-	-	-
<i>Lactuca seriola</i>	55.3	-	+	+
<i>Lathyrus pratensis</i>	33.3	-	+	+
<i>Lepidium virginicum</i>	16.9	-	-	-
<i>Lolium perenne</i>	21.8	-	-	+
<i>Lotus corniculatus</i>	27.1	-	+	+
<i>Lysimachia vulgaris</i>	27.5	-	-	-
<i>Medicago falcata</i>	20.8	-	+	+
<i>Myosotis palustris</i>	53.1	-	-	-
<i>Oenanthe aquatica</i>	n.d.	-	-	-
<i>Ononis repens</i> ssp. <i>spinosa</i>	13.3	-	-	-
<i>Origanum vulgare</i>	29.6	-	-	-
<i>Pastinaca sativa</i>	24.4	-	-	-
<i>Phalaris arundinacea</i>	25.0	-	+	+
<i>Plantago intermedia</i>	24.9	n.d.	-	-
<i>Plantago lanceolatum</i>	19.3	n.d.	-	-
<i>Plantago major</i> ssp. <i>major</i>	15.9	n.d.	-	-
<i>Poa annua</i>	89.3	+	+	+
<i>Poa pratensis</i>	21.1	-	+	-
<i>Poa triviale</i>	14.8	-	+	+
<i>Polygonum amphibium</i>	21.0	-	-	-

(Continues)

Table 1. (Continued)

Species	SLA (m ² kg ⁻¹ DM)	Stem	Gas film (presence-absence)	
			Leaf adaxial side	Leaf abaxial side
<i>Potentilla anserina</i>	23.0	+	-	+
<i>Potentilla reptans</i>	24.0	-	-	-
<i>Ranunculus acris</i>	26.4	+	-	+
<i>Ranunculus repens</i>	24.2	-	-	-
<i>Ranunculus sceleratus</i>	66.0	-	-	-
<i>Rorippa amphibia</i>	31.5	-	-	-
<i>Rorippa sylvestris</i>	15.3	-	-	-
<i>Rubus caesius</i>	26.8	-	-	-
<i>Rumex conglomeratus</i>	33.5	-	-	-
<i>Rumex crispus</i>	18.0	-	-	-
<i>Rumex obtusifolius</i>	22.1	-	-	-
<i>Rumex palustris</i>	61.4	-	-	-
<i>Rumex thyrsiflorus</i>	19.4	-	-	-
<i>Sagittaria sagittifolia</i>	24.4	-	-	-
<i>Salix cinerea</i>	16.8	-	-	+
<i>Salvia pratensis</i>	30.2	-	-	-
<i>Saponaria officinalis</i>	19.0	-	-	-
<i>Scutellaria galericulata</i>	58.6	-	-	-
<i>Sedum acre</i>	8.0	-	-	-
<i>Senecio jacobaea</i>	26.3	-	-	-
<i>Silene pratensis</i>	23.1	-	-	-
<i>Sium latifolium</i>	39.0	-	-	-
<i>Symphytum officinale</i>	21.1	-	-	-
<i>Tanacetum vulgare</i>	22.5	-	-	-
<i>Taraxacum officinale</i>	26.0	n.d.	-	-
<i>Thalictrum minus</i>	18.9	-	+	+
<i>Tragopogon pratensis</i>	23.1	-	+	+
<i>Trifolium dubium</i>	30.6	-	+	+
<i>Trifolium pratense</i>	25.3	-	+	+
<i>Trifolium repens</i>	18.2	-	-	-
<i>Veronica teucrium</i>	19.8	-	-	+
<i>Viccia cracca</i>	28.3	+	+	+

SLA, specific leaf area; n.d., not determined.

($n=1$). Once dried, samples were transferred directly for cooling in a desiccator containing silica gel and then weighed on a five-digit balance. SLA (m² kg⁻¹) was calculated from surface area and dry mass.

Cultivation of plants

Twenty-five species of terrestrial plants distributed along the flood gradient and with leaves of contrasting ability to form gas films when submerged (Table 1) were chosen for laboratory measurements of underwater net photosynthesis (P_N) and underwater dark respiration (R_D). Eleven species did not form a gas film when submerged, with seven species representing relevées with few flood occurrences and four species representing relevées with frequent flood occurrences. Fourteen species formed gas films when submerged, with nine species derived from relevées with few flood occurrences and five species derived from relevées with frequent flood occurrences. Plants were collected in the field in late spring and subsequently planted in pots (10 cm high and 6 cm wide) in a mixture of potting soil (DANMULD, Denmark; mixture of peat moss, cattle manure and bark mulch, containing: 45–65 g N m⁻³, 20–40 g P m⁻³, 15–40 g K m⁻³, 15–30 g Mg m⁻³ and >150 g Ca m⁻³) and sand (50/50, vol/vol). The pots were placed outdoors in plastic trays (60 cm wide and 100 cm long and 25 cm deep) in North Zealand, Denmark, and connected to an automatic irrigation system with tap water. The pots were watered twice in each 24 h cycle and drainage holes 2 cm above the bottom enabled excess water to drain out. After 8 weeks of growth during June and July, plants were used in measurements of underwater P_N , underwater R_D and gas film retention time.

Underwater P_N

The youngest fully expanded leaf was collected and cut with a razor blade into tissue segments of 1 to 2 cm² (except for *E. arvense*, here stem segments approximately 2 cm long were used). Underwater P_N was measured using the method described by Pedersen, Colmer, & Sand-Jensen (2013) with some modifications. The glass vials used for incubation were 25 mL, and two glass beads were added to each vial to ensure mixing as they rotated on a wheel within an illuminated water bath at 20 °C. Photosynthetically active radiation was 800 μ mol photons m⁻²s⁻¹ inside the vials, measured using a spherical light sensor (4 π US-SQS/L Wals, Effeltrich, Germany). The incubation solution (artificial floodwater) was based on the general purpose culture medium described by Smart & Barko (1985) and contained (in mol m⁻³): Ca²⁺, 0.62; Mg²⁺, 0.28; Cl⁻, 1.24; SO₄²⁻, 0.28. The dissolved O₂ concentration in the medium was set at 50% of air equilibrium, by purging the solution with 1:1 volumes of N₂ and atmospheric air. This procedure was applied, like in earlier studies (Colmer and Pedersen 2008), to prevent increase in O₂ above air equilibrium levels during the measurements, which might have led to photorespiration and thus decreased P_N [cf. Setter *et al.* (1989)]. Dissolved CO₂ was set at 180 mmol m⁻³ by adding 2.8 mol m⁻³ KHCO₃ and adjusting pH to 7.4 with HCl (Stumm and Morgan 1996).

Following incubations of known duration (from 60 to 175 min depending on P_N rates), dissolved O₂ concentrations in the solution were measured using a Clark-type O₂ mini-electrode (OX-500, Unisense A/S, Aarhus, Denmark) connected to a multimeter (MicroSensor Multimeter; Unisense

A/S, Denmark). The electrode was calibrated immediately before use in water at atmospheric equilibrium (20.6 kPa pO_2) and in anoxic water containing sodium dithionite (0 kPa pO_2). Dissolved O₂ concentrations in vials prepared and incubated in the exact same way as described previously, but with no leaf tissue, served as blanks. The projected area of each lamina segment was measured using a leaf area metre (CI-202, CID Bio-Science Inc., Camas, WA, USA) to enable expression of P_N rate per unit of surface area. Leaf tissues were then oven-dried until constant weight at 65 °C. Once dried, samples were transferred directly to a desiccator containing silica gel and then taken and weighed using a five-digit balance.

Underwater dark respiration (R_D) was measured as earlier but in complete darkness. Artificial floodwater was prepared to hold 10 or 40 kPa of pO_2 to determine R_D under restricted (10 kPa) or unrestricted (40 kPa) O₂ availability. The 10 kPa solution was prepared by purging half of the volume with atmospheric air and half with pure N₂ resulting in a pO_2 of approximately 10 kPa. The 40 kPa solution was prepared by purging 400 mL of incubation solution with pure O₂ and mixing 600 mL solution purged with pure N₂. As R_D rates are lower than P_N rates, incubation times for the R_D measurements were approximately 3 h.

Gas film retention time

Gas film retention time was measured on 13 of the 14 species with leaf gas films that were also used in measurements of underwater P_N and R_D . Four pots of each species were completely submerged in a 325 L outdoor aquarium filled with artificial floodwater during summer in North Zealand, Denmark. CO₂ was kept at ~250 mmol m⁻³ [2 mol m⁻³ alkalinity and pH 7.3, for chemical composition, see above (Stumm and Morgan 1996)], using a pH-controller (α -control, Dupla Aquaristik, Bielefeld, Germany) connected to a cylinder with pressurized CO₂. Each day, presence-absence of the leaf gas film on the same leaf was recorded by visual inspection (see previous text) at approximately 9:00 AM.

Data on species composition and flood patterns

The database from the study of Voesenek *et al.* (2004) was used. In brief, the database consists of 92 relevées (plots) ranging from 4 to 25 m², containing 72 plant species distributed across a flood gradient in the floodplains of the river Waal in the Netherlands. Each relevé has corresponding information on flooding frequency, inundation duration, substrate moisture, substrate type and rate of dehydration in addition to plant species composition and density. The elevation of each relevé was determined by levelling or by using detailed topographical maps and together with data on water level of the river (10 years average) the flooding regime of the relevées was established. On an average, the areas with the highest flood occurrences had 10 floods per year lasting up to 23 d (Fig. 1), whereas the areas with the fewest flood occurrences had 0.1 floods per year (one flood in a 10 year period) lasting only 2 d (Voesenek *et al.* 2004).

Statistical analyses

Detrended correspondence analysis (DCA) (Jongman, ter Braak, and van Tongeren 1995; ter Braak and Smilauer 2002) using the CANOCO V.4 software package (ter Braak and Smilauer 2002) was used to extract the main gradients in species community data. The analysis was run on semi-quantitative data including 72 species from 92 relevés. Data were not transformed before analysis. Spearman's rank correlation analyses (GraphPad Prism 5) between DCA axis 1 site scores and environmental variables were used to test the primary gradients at the study site.

GraphPad Prism 5 was used to analyse data and to draw figures. Kruskal–Wallis non-parametric tests with Dunn's multiple comparison *post hoc* tests were used to test for significant differences of the means of 'often flooded' and 'rarely flooded' plant species with and without gas films with regard to underwater P_N and underwater R_D .

RESULTS

DCA analysis of relevés and species distribution

DCA showed a strong gradient in species turn-over (Beta diversity) (Supporting Information Fig. S1). The gradient length along axis 1 was 6.6 (Supporting Information Table S1), indicating that the communities at the ends of the gradient did not share any species at all. The DCA explained 16.4% of the variation in species community data along axis 1, and an additional 12.4% of species data were explained on the three subsequent axes (Supporting Information Table S1). Supporting Information Fig. S1 illustrates the separation into two distinct clusters representing communities that are 'often flooded' and 'rarely flooded'. The strongest correlation between environmental variables and the primary gradient in species data (DCA 1) was flooding frequency (floods per year) (Fig. 1a) and flooding duration (d year^{-1}) (Fig. 1b) averaged over a 10 year period.

DCA 1 was based on species composition of the 92 relevés and was strongly negatively correlated to both flooding duration ($0.2\text{--}205 \text{ d year}^{-1}$, $r = -0.97$) and flooding frequency ($0.1\text{--}10$ floods per year, $r = -0.94$). Consequently, we used the words 'often flooded' and 'rarely flooded' on all subsequent figures with DCA 1 values on the horizontal axis to illustrate the compound effect of both flooding frequency and duration.

Presence-absence of leaf gas films

The main hypothesis was that leaf gas films is a flooding tolerance trait over-represented in habitats characterized by short floods, whether frequent or rare [*cf.* Winkel *et al.* (2011)]. Therefore, presence-absence of leaf gas films was surveyed for plant species found in the flood gradient (Table 1). We found that 41 of the 95 species tested were able to retain a gas film and with only one species overlapping with the already eight species known to form gas films (Introduction), the knowledge on occurrence of this trait in a natural vegetation was significantly expanded.

Figure 2 shows the percentage of species with leaf gas films in a given relevé along the flood gradient. We expected a local maximum along the flooding axis reflecting over-representation of gas films in environments with short floods. However, the relationship between flood occurrence and percentage of species with leaf gas films shows a continuous increase from 'often flooded' to 'rarely flooded' habitat reaching a maximum value of about 80% except for a single outlier (Fig. 2a). This relationship was strongly influenced by grasses that all possess gas films (Fig. 2b). Hence, if an area is dominated by grass species, leaf gas films will always be a common trait. However, if grasses were excluded from the dataset, a similar trend was found, that is, the percentage of gas film species still increased towards the 'rarely flooded' end of the gradient (Supporting Information Fig. S2). In the present data set, up to 66% of species in a given relevé were grass species, but the relative proportion of grasses differed along the flood gradient. In the 'often flooded' end of the gradient, several relevés had no grasses at all, whereas the middle of the flood gradient showed the greatest variation in percentage of grasses ranging from approximately 20 to 60%. In the most 'rarely

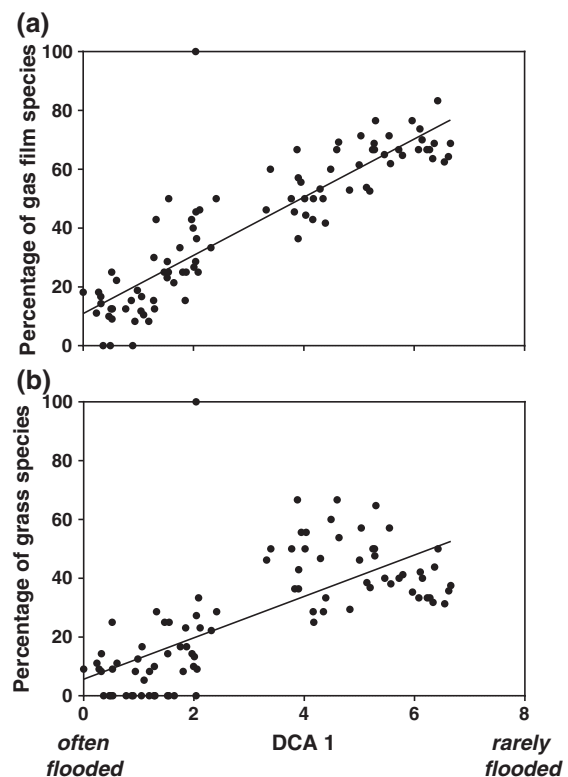


Figure 2. The percentage of species with leaf gas films in each relevé as a function of DCA 1 value ($n = 92$; $P < 0.0001$; $r = 0.9$; Spearman's rank correlation) in (a) and the percentage of grass species in each relevé as a function of DCA 1 value ($n = 92$; $r = 0.76$; $P < 0.0001$; Spearman's rank correlation) in (b). DCA 1 values are significantly correlated with number of flooding events and total inundation duration per year; low DCA 1 values correspond to frequent or long-term flooding (up to 10 floods per year, duration on average between 9 and 23 d) (Figure 1). Relevés with high DCA 1 values can experience as little as one flood, which lasts up to 5 d, in a 10 year period.

flooded' end of the flood gradient, the proportion of grasses again tended to decline (Fig. 2a).

Specific leaf area

As a second hypothesis, we expected that thin-leaved species (high SLA) should dominate the 'often flooded' end of the flood gradient. We use SLA as a proxy for leaf thickness (high SLA, thin leaves) albeit thick leaves would have higher SLA if the tissue porosity is high, but such leaves would still benefit from a high SLA during complete submergence, because this leaf trait will reduce total resistance to gas exchange with the floodwater. Whether it is flooding occurrence or availability of water that controls SLA in the 'often flooded' end of the flood regime is hard to separate, but the fact remains that high SLA increases gas exchange with floodwater and thus submergence tolerance (Mommer, Pedersen, and Visser 2004; Mommer *et al.* 2005). Such a relationship between flood occurrence and SLA was indeed present. Figure 3 shows the average SLA of species in each relevé along the flood gradient, and SLA displays a strong correlation with flood occurrence, that is, high SLA (thin or more porous leaves) in the 'often flooded' end of the gradient and thicker leaves in the 'rarely flooded' end.

Leaf gas film retention time

The thin gas film is only retained as long as the leaf cuticle remains superhydrophobic, and consequently, we tested if gas film retention time differed amongst the 25 selected species. Of these, 14 possessed leaf gas films when submerged into water, but only 13 were included in the analysis of gas film retention time. Gas film retention time varied greatly as one species, *Potentilla anserina*, lost the gas film on the day

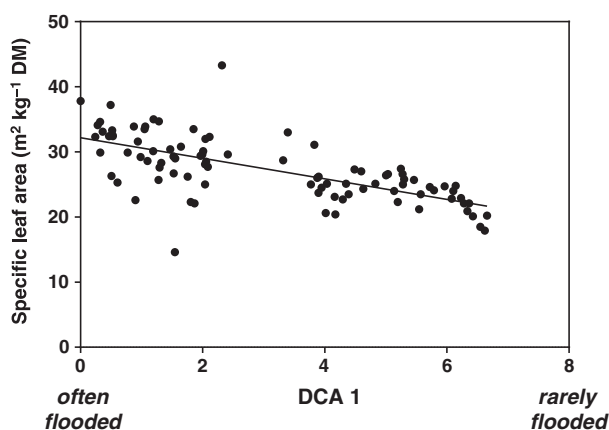


Figure 3. Specific leaf area (first fully developed leaf) of species found in each relevé versus DCA 1 values. SLA values are given as a mean based on species present in a given relevé. There was a significant negative correlation between specific leaf area and DCA 1 value ($n=92$; $r=-0.72$; $P<0.0001$; Spearman's rank correlation). DCA 1 values are significantly correlated with flooding events, inundation duration, soil moisture and soil dehydration; low DCA 1 values correspond to frequent or long-term flooding (Figure S2).

it was submerged, while the longest-lasting gas film was retained in *Agrostis stolonifera* for more than 11 d (Table 2). Moreover, eight of the 12 remaining species retained gas films for more than 7 d of complete submergence. There was no statistically significant difference in mean gas film retention time between the five species from the 'often flooded' and the eight species from the 'rarely flooded' part of the flood gradient. Thus, gas film retention time does not seem to be correlated with the compound stress exerted by flooding frequency and duration.

Underwater net photosynthesis (P_N)

Underwater P_N of the 25 selected species varied between 0.03 and $1.06 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ (Fig. 4a). When divided into 'often flooded' and 'rarely flooded', there was no significant difference in underwater P_N (unpaired t -test, $P=0.0556$, $n=25$, Fig. 4a). When leaf gas film was included as a parameter, highly significant differences ($P<0.001$) were found between 'often

Table 2. Presence-absence of leaf gas films, gas film retention time and habitat of 25 species of terrestrial plants used in laboratory experiments of underwater photosynthesis, underwater dark respiration and gas film retention time during submergence

Species names	Gas film	Leaf gas film retention time (days)	Habitat
<i>Potentilla anserina</i>	+	0	Often flooded
<i>Trifolium pratense</i>	++	1	Rarely flooded
<i>Chenopodium glaucum</i>	++	3	Often flooded
<i>Equisetum arvense</i>	++	3	Rarely flooded
<i>Euphorbia cyparissias</i>	++	3	Rarely flooded
<i>Arrhenatherum elatius</i>	++	8	Rarely flooded
<i>Dactylus glomerata</i>	++	8	Rarely flooded
<i>Phalaris arundinacea</i>	++	8	Often flooded
<i>Alopecurus pratensis</i>	++	9	Rarely flooded
<i>Festuca rubra</i>	+	9	Rarely flooded
<i>Glyceria maxima</i>	++	9	Often flooded
<i>Lolium perenne</i>	+	9	Rarely flooded
<i>Agrostis stolonifera</i>	++	11+	Often flooded
<i>Eryngium campestre</i>	++	NA	Rarely flooded
<i>Anthriscus sylvestris</i>	-	No gas film	Rarely flooded
<i>Bidens tripartita</i>	-	No gas film	Often flooded
<i>Cerastium arvense</i>	-	No gas film	Rarely flooded
<i>Chenopodium rubrum</i>	-	No gas film	Often flooded
<i>Galium molle</i>	-	No gas film	Rarely flooded
<i>Plantago lanceolatum</i>	-	No gas film	Rarely flooded
<i>Potentilla reptans</i>	-	No gas film	Rarely flooded
<i>Rorippa amphibia</i>	-	No gas film	Often flooded
<i>Rumex palustris</i>	-	No gas film	Often flooded
<i>Rumex thyrsoiflorus</i>	-	No gas film	Rarely flooded
<i>Taraxacum officinale</i>	-	No gas film	Rarely flooded

Leaf gas films of the first fully expanded leaf were either present on both the abaxial and the adaxial side of the leaf (++), present on just one side (+) or absent (-). NA = not available. Leaf gas film retention time was measured by observing four replicate plants of each species completely submerged in an aquarium in an outdoor facility in Denmark during the month of August.

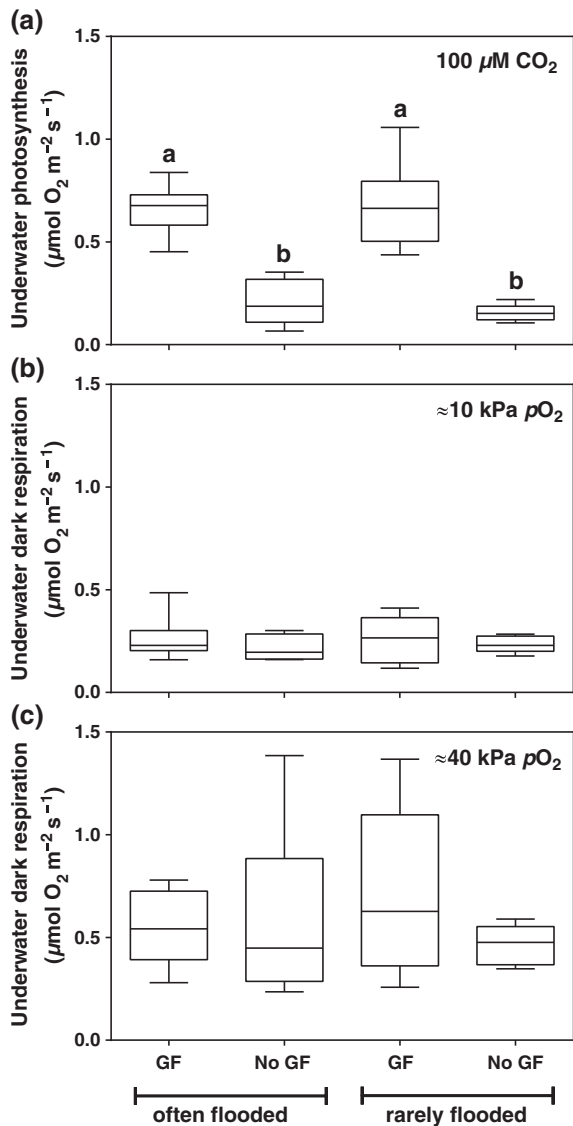


Figure 4. Underwater net photosynthesis (a), underwater dark respiration at 10 (b) and 40 (c) kPa pO_2 of leaves of 25 species of terrestrial plants divided into ‘often flooded’ (median DCA 1 value of 1.3) and ‘rarely flooded’ (median DCA 1 value of 5.1) species with or without leaf gas films (GF) when submerged. In the box-whisker plots, the horizontal line indicates the median, the box covers 50% of the observations and the bars indicate the data range (minimum to maximum). Letters in (a) indicate significant differences between the groups of species from mud flats or wet soils communities (Kruskal–Wallis with Dunn’s *post hoc* test, $n = 25$, $P < 0.001$), whereas in (b) and (c), there were no significant differences between the groups.

flooded’ species with and without leaf gas films, and between ‘rarely flooded’ species with and without leaf gas films, but not between the ‘often flooded’ and ‘rarely flooded’ groups of species with or without leaf gas film (Fig. 4a). Overall, species with leaf gas films had on average 3.8-fold higher underwater P_N per leaf area than species without leaf gas films, regardless of habitat.

Underwater P_N per species was used to calculate the mean species underwater P_N for each relevé in which some or all of

these 25 species were present (i.e. if 12 of the 25 species were present in a given relevé, their mean underwater P_N rates were used to specify the relevé’s underwater P_N). Underwater P_N of species with leaf gas films (Fig. 5a) were significantly correlated to flood occurrence with higher values of P_N in the ‘often flooded’ end of the gradient resulting in the relevés having higher mean species underwater P_N . Underwater P_N of species without leaf gas films also had a slight positive correlation with flood occurrence (Fig. 5a). Average underwater P_N of all species in the habitats showed a negative correlation with flood occurrence (Fig. 5b), probably because of higher abundance of species with leaf gas films (which have higher underwater P_N) in the ‘rarely flooded’ end of the flood gradient (Fig. 2). The percentage of species with leaf gas films was 19% in the ‘often flooded’ end of the gradient but 67% in the ‘rarely flooded’ end.

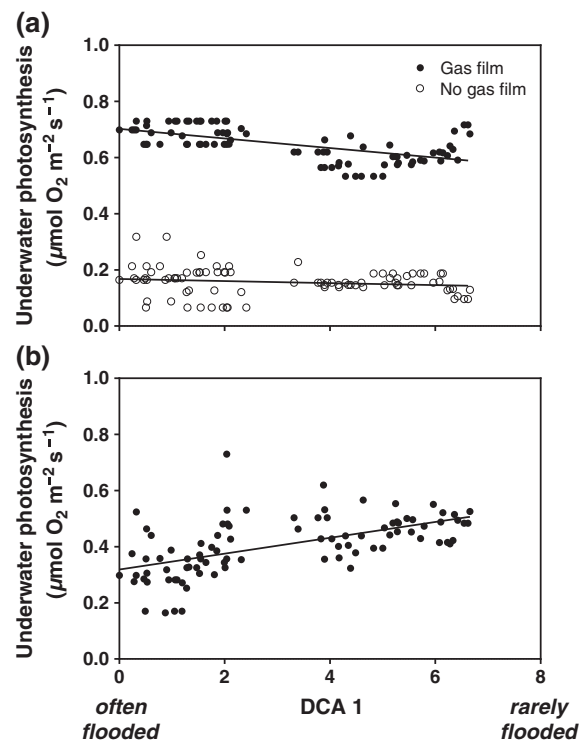


Figure 5. Mean underwater net photosynthesis of plant species in each relevé versus DCA 1 values divided into species with leaf gas films [panel (a), black circles, $n = 85$; $r = -0.59$; $P < 0.0001$; Spearman’s rank correlation] or without leaf gas films when submerged [panel (a), open circles, $n = 88$; $r = -0.25$; $P = 0.021$; Spearman’s rank correlation]. Mean underwater net photosynthesis of all species present in each relevé no longer divided into with or without gas film (average underwater P_N of species present in a relevé with and without leaf gas films) versus DCA 1 value [panel (b), $n = 90$; $r = 0.64$; $P < 0.0001$; Spearman’s rank correlation]. DCA 1 values are significantly correlated with flooding events and inundation duration; low DCA 1 values correspond to frequent or long-term flooding (Supporting Information Fig. S2). Because some relevés had no species in them when divided into ‘with’ or ‘without leaf gas films’, n is lower in panel (a) than in panel (b).

Underwater dark respiration (R_D)

Underwater R_D was measured both at 10 kPa (expected to limit R_D) and at 40 kPa pO_2 (expected to saturate R_D). At limiting pO_2 , there was no significant difference in underwater R_D between 'often flooded' and 'rarely flooded' species neither with nor without leaf gas films (Fig. 4b).

Underwater R_D measured at high pO_2 for leaves of plants in the 'rarely flooded' end of the flood gradient was 1.3-fold higher than in leaves from plants in the 'often flooded' end (Fig. 4c). On average, 'rarely flooded' relevées had 1.3-fold higher underwater R_D compared with 'often flooded' relevées. Moreover, there was a significant correlation between flood occurrence and mean underwater R_D of leaves of plant species in each of the relevées (Fig. 6). The relationship between underwater P_N and underwater R_D showed a strong positive correlation both at limiting pO_2 for R_D and at high pO_2 (Supporting Information Fig. S3); R_D was twofold higher at 40 kPa pO_2 compared with rates at 10 kPa pO_2 . We conclude that species from areas with frequent, long-lasting floods had lower rates of underwater R_D than those from rarely flooded sites; these differences in R_D expressed on an area basis reflect that leaves were thinner for the species towards the more often flooded end of the gradient.

DISCUSSION

The flood gradient examined in the present study showed a significant change in plant species composition from the 'often flooded' to the 'rarely flooded' end of the gradient. This is expected to reflect filtering of species with plant traits that enhance survival during submergence such as flooding-induced shoot elongation related to water depth and duration of floods in a river floodplain (Colmer and Voesenek 2009; Voesenek and Bailey-Serres 2015). In addition, life-history traits are assumed to be important in the distribution and abundance of

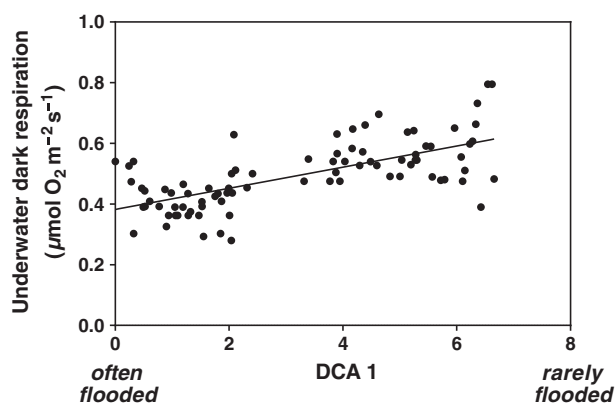


Figure 6. Mean underwater dark respiration of species in each relevé versus DCA 1 value ($n = 85$; $r = 0.66$; $P < 0.0001$; Spearman's rank correlation). Underwater dark respiration (R_D) values obtained at 40 kPa pO_2 were used (Fig. 3). DCA 1 values are significantly correlated with flooding events, inundation duration, soil moisture and soil dehydration; low DCA 1 values correspond to frequent or long-term flooding (Supporting Information Fig. S2). Underwater R_D values were not obtained for three of the 25 species investigated resulting in five relevées with no data for R_D .

plant species in flood-prone environments. The present study demonstrated that larger SLA (i.e. thinner or more porous leaves) was strongly positively correlated to flood occurrence (a combination of frequency and duration), so that species, overall, had thinner leaves in the 'often flooded' end of the gradient thereby reducing total resistance to gas exchange with floodwater (Madsen and Sand-Jensen 1991). Although it has been demonstrated in laboratory studies (Colmer and Pedersen 2008; Pedersen *et al.* 2009) and field investigations (Winkel *et al.* 2011; Winkel *et al.* 2013) that leaf gas films also greatly enhance gas exchange with the floodwater, leaf gas films as a species-specific trait did not show the hypothesized positive relationship with flood occurrence (up to a limit; i.e. frequent but relatively short duration floods during which gas films would be expected to be retained) for the floodplain species studied here.

Leaf gas films

Gas film retention during submergence varied from 1 to 11 d for the 13 species tested, which were selected from the larger group observed to initially retain such a film (Table 2). Moreover, there was no statistical difference between duration of gas film retention amongst species that were frequently flooded and species that were flooded less often. Gas film retention has previously only been evaluated for four genotypes of rice in a field situation where even the most submergence tolerant genotype, FR13A, retained its leaf gas films for at most 6 d of submergence (Winkel *et al.* 2014), while *M. siculus* retained the leaf gas film only for 3 d in a greenhouse experiment (Teakle *et al.* 2014). Here, the median gas film retention time was 8 d for frequently flooded species as well as rarely flooded species. Interestingly, the leaves of *A. stolonifera* retained gas films for more than 11 d (Table 2). The preferred habitat of *A. stolonifera* is in the 'often flooded' end of the gradient, and relevées in which this species occurred would on average experience three floods per year with up to 50 d of inundation in total (Fig. 1). *A. stolonifera* possesses a relatively resilient leaf gas film that would enhance CO_2 uptake for photosynthesis (Fig. 4a, Colmer and Pedersen 2008; Raskin and Kende 1983) and O_2 entry for dark respiration (Fig. 4b, Colmer and Pedersen 2008; Pedersen *et al.* 2009; Verboven *et al.* 2014) during times of complete submergence. It is thus likely that the leaf gas film trait is of functional importance to certain species along the studied flood gradient.

As earlier hypothesized, we expected an over-representation of the leaf gas films trait in plant species along the flood gradient. Flood duration per event varied on average from 9 to 23 d in the flood-prone areas to 0–2 d in the less flooded areas, whereas gas film retention time lasts from less than 1 to 11 d (evaluation of 13 species with leaf gas films; Table 2), so a possible over-representation (or 'cluster') of species with this trait (assessed as initial presence) along the flood gradient might have been evident, but it was not. Thus, we could not establish a correlation between presence of leaf gas films and the distribution of plant species along the flood gradient. This result does not preclude that leaf gas films might benefit species in habitats that are frequently flooded for a relatively short time, as demonstrated in other studies (Winkel *et al.* 2011).

Except for the initial DCA analysis that served to arrange the relevées according to flooding frequency and duration, we omitted using the species density data and restricted all subsequent analyses to species presence-absence. We selected this approach in order to compensate for some of the ecological processes leading to dominance (or disappearance) of species following a catastrophic event such as flooding. When a flood event results in complete submergence, possession of traits such as, for example, petiole elongation and/or formation of acclimated leaves with high SLA as shown for *Rumex palustris* (Voisenek *et al.* 2003a; Mommer *et al.* 2005) and leaf gas films as shown for rice and *M. siculus* (Pedersen *et al.* 2009; Teakle *et al.* 2014) are likely to result in enhanced survival. However, when the water recedes and the soil dries up, traits associated with a terrestrial lifestyle become more favourable, while traits associated with flooding are disadvantageous according to cost-benefit principles. Ultimately, traits important for interspecific competition, sensitivity to grazing and adaptation to drought, for example, leaves with low SLA (Violle *et al.* 2011; Baastrop-Spohr *et al.* 2015), may influence species density and presence-absence of various species. Thus, as time progresses following a flood event, the impact of the flood upon the species composition diminishes.

Plant performance in non-flooded conditions following the last flood is probably of less importance in the 'often flooded' than in the 'rarely flooded' areas. In the 'often flooded' areas, the relevées experience up to 10 floods per year, and the vegetation may be inundated annually for 235 d (Fig. 1). We propose that relevées located in such a flooding-dominated environment would host vegetation that is strongly adapted to submergence and encompass species that are able to produce aquatic leaves (e.g. *Mentha aquatica*, *Myosotis palustris* and *Limosella aquatica*) which, in fact, was the case (data not shown). In contrast, the relevées located in the 'rarely flooded' areas may only experience a flood once every 10 years and become completely submerged for only 2 d (Fig. 1). Here, the flood event is unlikely to have any impact on species composition at all. Moreover, a complicating factor for the present investigation is that these typical grassland relevées are also grazed by cattle and sheep, and for that reason, grass species are benefitted. All grasses tested so far possess leaf gas films when initially submerged, driving the negative correlation between flood occurrence and percentage of species with gas films (Fig. 2) and possibly occluding a localized leaf gas film species over-representation (or 'cluster') along the flood gradient.

Underwater P_N

Our study has cemented the role of leaf gas films as a trait enhancing gas exchange between leaf and floodwater for a much larger number of species than previously assessed, and for the first time the beneficial role was demonstrated for non-wetland plants. Regardless of position on the flood gradient, leaf gas films enhanced underwater P_N by 3.7-fold to 4.4-fold (Fig. 4a), showing that leaf gas films operate equally well whether possessed by a plant from a wetland or from a drier habitat. The underwater P_N rates obtained by these leaves with gas films at 180 μM CO_2 resembled those of aquatic,

homophyllous plants measured at 90 to 400 μM CO_2 and were only surpassed by aquatic leaf-types of heterophyllous amphibious plants (Sand-Jensen, Pedersen and Nielsen 1992). In contrast, the barely positive rates of leaves of the terrestrial dryland plants without gas films are the lowest underwater P_N values observed to date and were only 46% of those of the terrestrial wetland plants previously studied (Sand-Jensen *et al.* 1992). Nevertheless, all species tested in the present study showed positive underwater P_N at 180 μM CO_2 , which is in the lower end of the CO_2 range encountered in overland floods (Colmer *et al.* 2011). Higher external CO_2 would probably enhance rates, but whether these plants are primarily CO_2 or light limited in the field during a flood is uncertain and depends on several conditions, for example, incident radiation, canopy density, floodwater clarity and CO_2 concentration (Pedersen *et al.* 2013). In the context of surviving a flooding event, even a modest underwater P_N can contribute to carbohydrate conservation and O_2 supply to shoot and roots (Mommer *et al.* 2004; Colmer and Voisenek 2009; Pedersen *et al.* 2009).

Underwater P_N shows an interesting pattern for each relevé on the flood gradient. Separating species into gas film and non-gas film species resulted in a negative correlation between flood occurrence and underwater P_N , although the relationship is less strong for species without gas films (Fig. 5a). This relationship for both gas film and non-gas film species is possibly driven by the relationship of SLA and flood occurrence (Fig. 3). Thicker leaves of species occurring in the 'rarely flooded' relevées also had lower gas exchange rates and thus a lower underwater P_N . However, when the analysis was performed including all species, the relationship changed completely, and 'rarely flooded' relevées now had higher underwater P_N than 'often flooded' relevées. This relationship is due to the increasing number of species with leaf gas films in the 'rarely flooded' end of the flood gradient (Fig. 2b), and because the trait of having leaf gas film is effective in underwater gas exchange (Colmer and Pedersen 2008), underwater P_N for these 'rarely flooded' relevées become high.

Specific leaf area

Thin leaves (high SLA) is another trait that confers survival during submergence, because such leaves reduce the total resistance to gas exchange with the floodwater (Madsen and Sand-Jensen 1991), as internal diffusion path lengths are shorter in distance. Moreover, thick leaves (low SLA) confer drought tolerance (Brock and Galen 2005), as thick leaves with a low surface:volume quotient loose lower proportions of the water pool per unit of time. This relationship between water availability (including excess water) and SLA is demonstrated in the present study by the positive correlation between SLA and flood occurrence (Fig. 3). The median SLA value of species occurring in 'often flooded' relevées is 30 $\text{m}^2 \text{kg}^{-1}$, which is the same as for the terrestrial wetland species in the study of Sand-Jensen *et al.* (1992). However, the study of Sand-Jensen *et al.* (1992) also included more aquatic species (amphibious plants and aquatic plants) with a median SLA of 92 $\text{m}^2 \text{kg}^{-1}$, which is beyond the range covered by the present study that

focuses on terrestrial plants. The strong relationship between flood occurrence and SLA was also demonstrated by Violle *et al.* (2011), covering a flood gradient similar to the one in the present study and also with a similar range in SLA [approximately 15 to 35 m² kg⁻¹ (Violle *et al.* 2011)]. Thus, it seems that SLA is a trait that is maintained in the vegetation community following a flood event; apparently, ecological processes (e.g. inter-specific competition and grazing) that could change species composition (presence or absence only was considered here) following a flood event were not sufficiently strong to change the strong relationship between SLA and flood occurrence.

CONCLUSION

We have shown in this study that species growing under conditions of high flood occurrence possess thinner or more porous leaves (higher SLA) than those growing in the 'rarely flooded' end of the flood gradient. This finding was anticipated, as such leaves minimize the total resistance to gas exchange when submerged. In contrast, the trait 'leaf gas films' did not show the hypothesized relationship of being most common in plants in areas with relatively short but frequent floods (that is, between the 'often flooded' and 'rarely flooded' ends of the flood gradient). Instead, leaf gas films were more common amongst species in the 'rarely flooded' relevées. This relationship was driven by the increased presence of grasses that all have superhydrophobic cuticles. The dominance of grasses in the 'rarely flooded' end of the gradient was probably stimulated by grazing. We propose that traits with ecophysiological significance can be diluted following a flood event as a result of the subsequent processes leading to a specific species composition of a relevé. In order to better evaluate leaf gas films as a submergence tolerance trait, we suggest investigating better-defined flood gradients (e.g. to enable distinguishing between winter and summer floods) and couple species composition with detailed studies of leaf gas film retention time.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Results of Detrended Correspondence Analysis (DCA) of 92 relevées hosting 72 species of plants from a natural flood gradient. For detailed information on sampling procedures see Voeselek *et al.* (2004).

Figure S1. Result of Detrended Correspondence Analysis (DCA) of 92 relevées hosting 72 species of plants from a natural flood gradient. The relevées group in two distinct clusters. For detailed information on sampling procedures see Voeselek *et al.* (2004).

Figure S2. The percentage of non-grass species (total number of non-grass species was 56) with leaf gas films in each relevé as a function of DCA 1 value ($n = 91$; $P < 0.0001$; $r = 0.76$; Spearman rank correlation). Number of species with leaf gas films was positively correlated to DCA 1 value.

Figure S3. Mean underwater dark respiration (RD) versus mean underwater net photosynthesis in each relevé for RD obtained at 10 kPa pO₂ ($n = 84$; $r = 0.43$; $P < 0.0001$; Spearman rank correlation) or at 40 kPa pO₂ ($n = 85$; $r = 0.43$; $P < 0.0001$; Spearman rank correlation).