SITE SPECIFIC STRESS & SEEDLING SETTLEMENT SUCCESS

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A LABORATORY APPROACH

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CONTENTS

INTRODUCTION

At the moment there is a new trend detectable in the Dutch reserve management. This is the development of new natural areas on former agricultural lands with high habitat creation potential, so-called 'nieuwe natuur' (new nature). This trend was initiated in 1988 with the presentation of 'Plan Ooievaar' (plan stork) with as main objective to develop dynamic river floodplains in former farmlands alongside the great rivers of the Rhine-delta. This trend has almost changed into a frenzy about the creationability of (botanically high-valued) natural areas on almost every site. Nowadays also a bad local farming economy (land surplus) or the proximity of built-up area (recreation demand) have become two main factors involved in site-choice for habitat creation (Baalen 1995, Tooren 1995, Knol 2001)). Other reasons, like water-storage can be a reason for nature development as well (Knol 2001). For biologists, an interesting consequence of this priority-shift is that research is required on quite unexpected sites, to uncover the natural potential of such an area.

One of the areas that is subject to habitat creation, and where this project is referring to, is the area of central Groningen around the drainage canal Duurswold, stretching from Kolham in the south-west to the Schildmeer in the north-east (Diggelen 2002).
In this area three main soil types are distinguished from south to north: 'Dalgrond' (exploited bogs, Eolian sand depositions surfacing), peat, and clay upon peat (clay layer < 1m thick) (Bosatlas 1988). Towards the north-east the concentration of chloride ions in the groundwater increases from < 3 mg/l up to 5000 mg/l. Sometimes concentrations as high as 8000 mg/l have been found (Bosatlas 1988). Due to the marine origin of the clay layer, not only relatively high concentrations of chloride are found, but also sulfate is abundantly present in the surface water. Seawater is rich in sulphuric compounds. Moreover the clay is rich in iron, sometimes visible as rusty stains (Fe_xO_x).

This research is done in the light of (re-)introduction of species (Groenendael 1998). The following factors play an important role in determining whether a certain species can settle successfully. 1) How far away is the nearest population of the species? 2) How successful is the species in dispersing its seed. 3) How many seeds does the mother plant produce? 4) How well germinates the seed and 5) How well can the seedling survive and reproduce in a specific situation.
This experiment refers to a bigger project where seed of a specific vegetation is brought into the area of habitat creation. This overcomes distribution problems. We assume that germination itself will not be stressed. That leaves factors 1 until 4 outside the main question. Therefore this experiment will focus on the survival of seedlings under different stress-treatments. Stress factors that the seedlings will probably face when introduced to the site of habitat creation.

Two 'Natuurdoeltypen' (vegetation objectives) (Bal 2001) considered suitable for this moist area will be looked at in this research. The *Calthion palustris* with the target species *Caltha palustris*, and the *Alopecurion pratensis* with the target species *Sanguisorba officinalis*. From both vegetations, the more common species *Ranunculus acris* will be considered in this experiment. Both vegetation types mentioned above thrive on periodically inundated and productive soils (Schaminée 1996), as is occur in some parts of the central Groningen area.

In regularly inundated soils oxygen depletion occurs (Armstrong 1975). This causes soil micro-organisms to switch from oxygen to another, less efficient, electron acceptor to use for respiration, like sulfate (richly abundant in some soils in the area and in the surface water). At

the reduction of sulfate, sulfide (H_2S, HS^- and S^{2-}) is formed. These compounds are toxic to plants (Koch 1989, Kok 2002) and may probably play an important role in seedling establishment. Also too high chloride levels can cause stress in plants. Another factor that could be of influence, is when seedlings have to break through an existing canopy to reach the sunlight. This necessary for photosynthesis, and forth, growth & secure settlement. Decrease in biomass has been found when plants were shade-stressed (Kotowski 2001, Rees 1995). A combination of these stress factors may pose an even greater complication to settling seedlings, but effects are largely unknown.
The ability of plants to cope with stress may explain the abundance or rarity of a species.

HYPOTHESES

Shading, high chloride levels, high sulfate levels or a combination of two or all of these factors will have a negative effect on the development of seedlings (1) and thus on their ability to settle in an area of habitat creation. However, of the used species, the ones that are rare are expected to be affected to a greater extend by these factors than the more common one (2).

MATERIALS & METHODS

PLANTS

Seeds of all used species were gathered in the same year (2002) in the wild. Forth they were all germinated in the same diurnal (light/dark, 25 °C/15°C) nursery cupboard. After germination, seedlings were retarded at 4°C, awaiting planting into the soil cores when the experiment started.

SPECIES

Caltha palustris, typical species from the *Calthion palustris*. Seeds were obtained in 2002 from existing population around the Drentsche Aa, Netherlands.

Sanguisorba officinalis, typical species from the *Alopecurion pratensis*. Seeds were obtained in 2002 from existing population around the Reest river, Netherlands.

Ranunculus acris. Common species, occurring in both plant communities mentioned above, and many others. Seeds were obtained from existing population in France

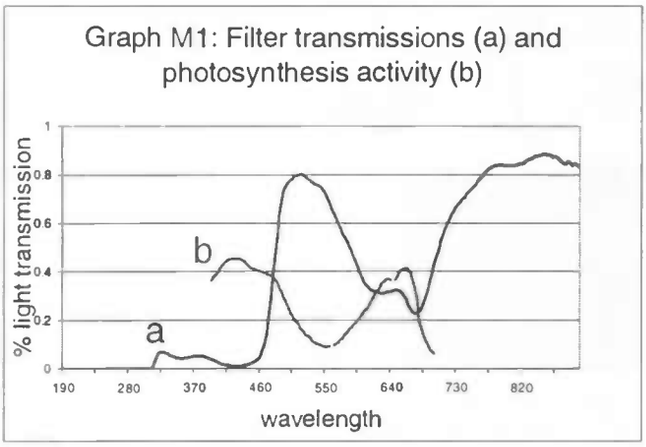
SOILS

The soils used in the experiment originate from the nature development site. Three types of soils are used: sand, peat and clay; the three main soils present in the midden Groningen reserve. They were excavated as monolithic soil cores, sustaining the vertical structure, and used as such in the experimental set-up. The dimensions are 10cm (depth) by 7cm (width) and they are contained in PVC cylinders. Like in the field situation, the plants are grown on the topside of these soil cores.

AREA

The midden Groningen reserve is an area of Pleistocene and Holocene sediments. The southern parts are mainly Pleistocene sandy soils, often subsoil's of exploited peatlands, whereas the northern part consists of Holocene marine clay sediments. In the middle part, peat is deposited. Parts of the area are inundated in winter, most with chloride- sulfate- and nutrient-rich surface water, but some with rainwater (Bosatlas 1988).

THE SHADE-TREATMENT



Graph M1.
In this graph the light permeability of the filter (LEE 121), used for the shading treatment, is shown, together with the curve of photosynthesis activity per wavelength.

The transmission values are measured with the spectrophotometer of the plant-physiology department of the RUG. This curve shows the percentage of light that is transmitted by the filter for every wavelength.
This graph shows that the wavelengths that are most used by the photosynthesis process are most intercepted by the filter, thus simulating a canopy of photosynthesising leaves. These wavelengths are roughly around 430 nm (blue light) and 650 nm (red light).

THE CHLORIDE-/ SULFATE-TREATMENT

The plants were given combined treatments with low and high concentrations of chloride and sulfate solutions in their irrigation water, resulting in four different treatments (see table M1).

Table M1. Ion concentrations (mM) of the different treatments. Note the levels of chloride and sulfate.

Ion	**Control -/-**	**Sulfate s/-**	**Chloride -/c**	**Combination s/c**
Ca^{2+}	0.72	1.22	1.32	1.82
Mg^{2+}	0.12	0.62	1.22	1.72
K^{+}	0.04	1.04	1.74	2.74
Na^{+}	0.46	1.46	3.16	4.16
Cl^{-}	0.55	0.55	8.35	8.35
SO_4^{2-}	0.11	2.11	0.11	2.11

Table M1. The high and low concentrations of chloride and sulfate are consistent with the outer boundaries of concentrations found in the field. The control treatment contains roughly the same amounts of ions as a mixture of groundwater (ion-rich) and rainwater (ion-poor). The concentrations of the cations in the sulfate- and chloride-treatments are dependent on the concentration of the anions of chloride and sulfate. It was persuaded to use even amounts of four different sulfate or chloride salts, to get an even distribution of the cations, thus avoiding an artefact by extreme cationconcentrations.

MATERIAL SET-UP

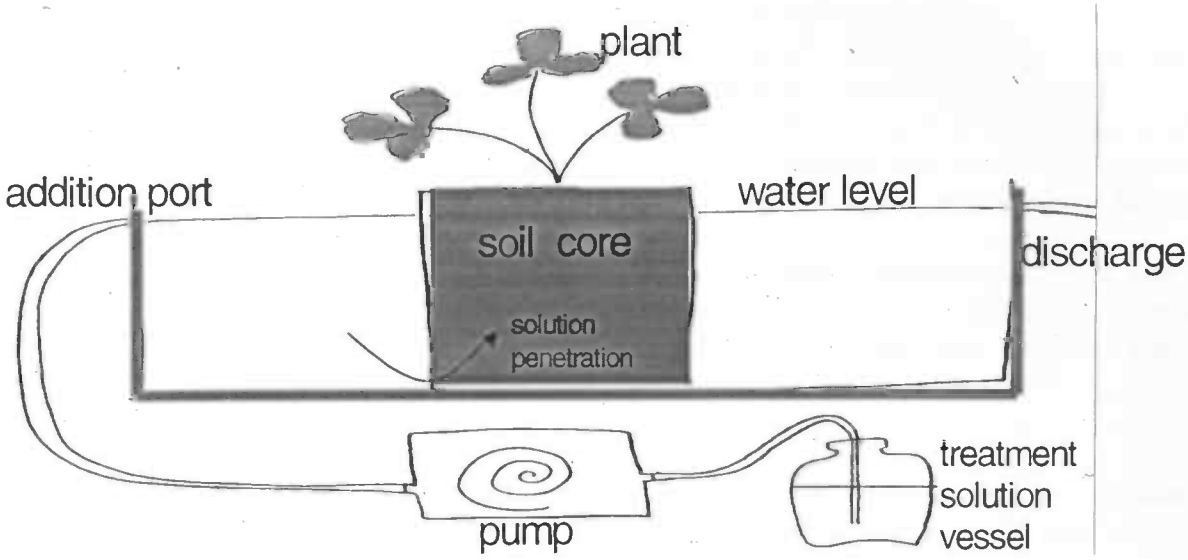


Fig. M1. Solution flow in experiment.
Vessels with a specific sulfate/chloride solution are attached to a pump that pumps the solution into the container where the soil cores with test plants are put. The water level inside the container is one cm below the soil surface. In this way it is made sure that the used soils are waterlogged and penetrated with the solution. The soil cores are fully enclosed on the sides by the PVC-cylinder. They are put on a fabric plaid, to make it possible for the solution to penetrate through the bottom. Forth the overage of water is discharged via a port, just one cm below the surface of the soil cores. The solution is refreshed by ten litres per week

Each container holds twenty-four pots of one soil type. It is exposed to one light regime, one sulfate and one chloride level. Three plant species are planted in a container, with one plant of one species per pot. So in total, eight plants of one species, divided over eight pots, grow in one container.
The experiment was carried out in two climate-chambers of which one was covered with the green filter, simulating overgrowing canopy. In each climate chamber twelve containers were placed, of which four contained soil cores of a similar type (i.e. sand). Of these containers with similar soil type, each one was treated with different solutions of sulfate and chloride.

PARAMETERS

In this experiment the most important measurements were done on shoot biomass (Hendry 1993), with discrimination of leaf and stem biomass, and the summa of these two: the total above-ground biomass. The biomass of a plant is considered a key parameter for measuring stress that a plant has had in the past lag of time, as well as the capabilities of an individual to cope with future stress. The root system was not measured for practical reasons. This implies that no root/shoot ratio could be measured. Apart from biomass the number of surviving plants in each treatment was counted. This too will say a lot about the way a species has been affected by a certain factor.

Apart from these phytoparameters there have been done measurements on the pH and the $[PO_4^{2-}]$ in the soil pore-water. pO_2, redox potential and $[S^{2-}]$ have been measured in the soil cores. Also organic matter, water, nitrogen and phosphorus contents have been surveyed in the soil types before use (see appendix).

MEASUREMENTS

Biomass of the tested plants was measured by drying the plants' leaves and stems, if possible separately, at 80°C in a forced ventilated oven for 24 hours (Hendry 1993), and weighing them on a three-decimals accurate balance.

Survival was measured simply by counting the number of surviving plants.

PH of the soil pore water was measured with a pH electrode (H_2O) and $[PO_4^{2-}]$ of this water was measured after addition of a molybdenum reagents with a spectrophotometer (*Spectronic genesys 5*). Soil pore water was obtained with so-called 'soil-samplers'. A water-permeable tube is stuck into the soil (after preparing a hole with a glass-rod) and attached to a airtight test-tube of 10 ml via a siring. By vacuuming the test-tube, soil pore water is slowly sucked up by the tube and deposited into the test-tube. Now it is possible to measure pH and use the solution for $[PO_4^{2-}]$ measurements. Due to the impermeable structure of clay, no data collection took place there.

Redox potential, pO_2 and sulfide concentrations were measured with electrodes (Microscale Measurements 2002, Adema 1997), attached to a datalogger. Four electrodes were used at the same time. One for redox and O_2 each, two for sulfide. Parameters were measured at a depth of 0 cm, -1 cm, -2 cm, -4 cm and -7 cm. Due to the impermeable structure of clay, no data collection took place there.

NESTED APPROACH

In this experiment, three different species were tested, growing on three different soil-types, exposed to three different stress-factors, each with one control-level. For every combination of treatments, eight plants were involved. The total number of treatments is 3(species) x 3(soil types) x 2(levels of light-intensity) x 2(levels of sulfate) x 2(levels of chloride)= 72.

STATISTICS (Zar 1999)

Since the experiment was designed as a nested approach, the first-choice statistical test would be the ANOVA. Every dataset that has one factor in common, e.g. high sulfate, can be subdivided into four subsets, containing all possible combinations of two levels of light and two levels of chloride (species and soils were not involved into the tests). The Analysis of Variance corrects for these differences. It also shows when there are significant specific differences between e.g. light/high chloride on one hand and dark/low sulfate on the other. On the other hand it requires that the tested data have a normal distribution or homogeneous variance. When these criteria don't match, a non-parametric test has to be performed. A Kruskal-Wallis is used when an ANOVA is not possible or allowed. This test does not take variations within the dataset into account, so one has to assume that the effects of the other factors are alike when combined with either one of the two treatments tested. E.g.: When one is testing the effect of sulfate, a high chloride level will have approximately the same effect on the plant when added solely, as it does in combination with a high sulfate level. All tests have been performed from numerical databases in the Microsoft statistics program SPSS. Homogeneity of variance for the datasets was tested with a Levinge test.

RESULTS

PLANT BIOMASSES AND SURVIVAL (TABLE R1, R2 AND R3)

CONTENTS

In these tables the effects of the different treatments on the three species are shown. In the first column the soil type is mentioned and in the second column the treatment. The second column is divided in three sets of treatments per soil type. Each set of treatments contains the expected stress factor treatment (upper row) plus its control treatment (middle row). In the lower row, the mean difference between the stressed and the control is shown, resulting in a negative value when the stress factor has a negative effect the parameter.
In the third, fourth and the fifth column, biomasses (g) of respectively leaf, stem and total aboveground tissue (forth referred to as 'total biomass') are shown for each treatment including the standard deviation. The 'n' in these columns represents the number of plants measured (max. 32). Although there are always 32 plants in each treatment, often there are less plants measured for biomass. This can be due to 1)omission of outlyers, 2)death losses or 3)dormant plants (These plants still show signs of life, like a small green bud at its growing point, but have developed to little biomass to measure) are also omitted. The different treatments contain aspects of both other treatments, e.g.: The shade-treatment population contains plants that were exposed to different chloride and sulfate levels, to the same extent as the light-treatment population does (see statistics).
In the seventh column the percentage of surviving plants (after 60 days) for each treatment is shown, including its standarddeviation. The 'n' in this column represents the populations, of eight plants each, grown under a treatment. In this case n=4 for each treatment. Populations are alike for the treatment that they are tested for, but differ for the other treatments, just as stated above. Statistics are performed on numbers, however results are shown as percentages.

STATISTICS

The biomass data are tested with both Kruskal-Wallis and ANOVA. Significant outcomes are marked with ^{k} for the Kruskal-Wallis test, and with * for the ANOVA. It was possible to perform ANOVA on most of the data, though not on all. Significance marks are placed between brackets (*), rather than omitted, when the tested dataset actually doesn't satisfy the conditions required for ANOVA. This is done so, because also Kruskal-Wallis shows significance in these cases.
The survival data are only tested with the non-parametric Kruskal-Wallis. Per ultimate combination of factors there is only óne population (n=1), so it is necessary to take the survival percentages of four populations together, to get one treatment testable (with n=4). This is why an ANOVA would not make sense in this case.
Significance levels are represented by the number of marks. E.g.: ^{kkk} = very sign. with Kr.-W.

COMBINATION EFFECTS

Although an ANOVA was performed, no effects were found for a combination of stress factors.

Table R1 (*Caltha palustris*)

		leaf biom. (g)	Stdev	n	stem biom. (g)	Stdev	n	total biom. (g)	Stdev	n	Survival(%)	Stdev (n=4)
sand	shade	0,011	0,011	7	0,007	0,004	7	0,019	0,016	9	56	36
	light	0,086	#	1	0,050	#	1	0,046	0,078	3	53	21
	mean diff.	-0,075			-0,043			-0,027			3	
	sulfate	0,061	0,036	2	0,032	0,026	2	0,046	0,056	5	41	26
	no sulfate	0,007	0,003	6	0,006	0,003	6	0,012	0,006	7	69	24
	mean diff.	**0,053*** k**			**0,026k**			**0,034***			-28	
	chloride	#	#	0	#	#	0	0,000	#	1	41	28
	no chloride	0,021	0,028	8	0,013	0,016	8	0,028	0,039	11	69	22
	mean diff.	#			#			-0,028			-28	
peat	shade	0,003	0,003	9	0,003	0,003	9	0,005	0,003	17	81	16
	light	0,003	0,004	8	0,004	0,004	8	0,004	0,005	16	84	12
	mean diff.	0,000			0,000			**0,001k**			-3	
	sulfate	0,003	0,004	6	0,004	0,003	6	0,004	0,004	14	81	16
	no sulfate	0,003	0,004	11	0,003	0,004	11	0,004	0,005	19	84	12
	mean diff.	0,000			0,001			0,000			-3	
	chloride	0,003	0,005	7	0,004	0,004	7	0,005	0,005	13	84	12
	no chloride	0,003	0,003	10	0,003	0,002	10	0,004	0,004	20	81	16
	mean diff.	0,001			0,000			0,001			3	
clay	shade	0,008	#	1	0,005	#	1	0,003	0,005	7	44	33
	light	0,053	#	1	0,019	#	1	0,009	0,019	13	44	44
	mean diff.	-0,044			-0,014			-0,005			0	
	sulfate	0,053	#	1	0,019	#	1	0,010	0,022	10	38	37
	no sulfate	0,008	#	1	0,005	#	1	0,003	0,004	10	50	40
	mean diff.	0,044			0,014			0,007			-13	
	chloride	#	#	0	#	#	0	#	#	0	13	18
	no chloride	0,030	0,031	2	0,012	0,010	2	0,007	0,016	20	75	10
	mean diff.	#			#			#			**-63k**	

Manova: *p<0,05 ; **p<0,01 ; ***p< 0,001
Kruskal - Wallis: k p<0,05 ; kk p<0,01 ; kkk p< 0,001
- sign indicates that no value is available

Table R1 (*Caltha palustris*).

Sand Sulfate has a positive effect on the biomass of leaves*** [k], stems[k] and the total aboveground plant***. Survival on the other hand seems to decrease with high sulfate[(no sign. levels)]. Again 'n' is quite small. In the chloride treatment, no plants were big enough to measure leaves or stems, but there seems to be a negative effect on survival and the total aboveground biomass. Shade seems to have a negative effect on the biomasses and a positive effect on survival, but this is not significant in any way. The number of tested plants is quite low.

Peat A small, but positive effect was found for shading on total biomass[k]. No effect was found for leaf and stem biomass or survival.

No significant effects were found for sulfate or chloride

Clay The measurable population was too small (n=1) to find any significant effect of the treatments, but in the chloride-treatments, survival[k] was affected negatively.

Table R2 (*Sanguisorba officinalis*)

		leaf biom. (g)	Stdev	n	stem biom. (g)	Stdev	n	total biom. (g)	Stdev	n	survival (%)	Stdev (n=4)
sand	shade	0,028	0,027	30	0,011	0,015	30	0,039	0,039	30	100	0
	light	0,020	0,018	29	0,005	0,007	29	0,025	0,023	29	91	19
	mean diff.	0,008			**0,006***			0,014			9	
	sulfate	0,021	0,020	28	0,008	0,013	28	0,030	0,030	28	91	19
	no sulfate	0,027	0,026	31	0,008	0,011	31	0,035	0,035	31	100	0
	mean diff.	-0,005			0,000			-0,005			-9	
	chloride	0,024	0,025	27	0,007	0,011	27	0,031	0,034	27	91	19
	no chloride	0,024	0,022	32	0,009	0,013	32	0,033	0,032	32	100	0
	mean diff.	-0,001			-0,001			-0,002			-9	
peat	shade	0,015	0,009	30	0,005	0,004	30	0,020	0,012	30	94	13
	light	0,020	0,011	29	0,004	0,003	29	0,024	0,012	30	97	6
	mean diff.	**-0,005***			0,000			-0,004			-3	
	sulfate	0,018	0,010	30	0,005	0,004	29	0,023	0,012	30	94	13
	no sulfate	0,018	0,011	30	0,004	0,003	30	0,022	0,013	30	97	6
	mean diff.	0,000			0,000			0,000			-3	
	chloride	0,015	0,010	29	0,003	0,002	28	0,018	0,011	29	91	12
	no chloride	0,021	0,010	31	0,005	0,004	31	0,026	0,012	31	100	0
	mean diff.	**-0,005*k**			**-0,002*k**			**-0,007*k**			-9	
clay	shade	0,013	0,008	26	0,027	0,117	26	0,039	0,116	27	88	10
	light	0,025	0,020	31	0,006	0,004	31	0,030	0,022	31	100	0
	mean diff.	**-0,012(**)kk**			0,021			**0,008k**			**-13k**	
	sulfate	0,017	0,010	28	0,026	0,113	28	0,042	0,111	29	94	7
	no sulfate	0,021	0,021	29	0,005	0,004	29	0,026	0,024	29	94	13
	mean diff.	-0,004			0,021			0,016			0	
	chloride	0,014	0,011	27	0,027	0,115	27	0,040	0,116	27	91	12
	no chloride	0,024	0,019	30	0,005	0,004	30	0,029	0,022	31	97	6
	mean diff.	**-0,011**kk**			0,021			0,011			-6	

Manova: *p<0,05 ; **p<0,01 ; ***p< 0,001
Kruskal - Wallis: k p<0,05 ; kk p<0,01 ; kkk p< 0,001
- sign indicates that no value is available

Table R2 (*Sanguisorba officinalis*).

Sand Shade seems to favour the biomasses of leaves, stems* and the total plant, as well as the survival. The effects of sulfate and chloride seem to be negative, rather than positive, on both biomasses and survival, but this is not significant.

Peat Shade has a negative effect on leaf biomass*, but no distinct effect on the biomasses of stems and the total plant, or survival.

Sulfate doesn't show any effect, but chloride affects the biomasses of leaves*^{k}, stems*^{k} and the total plant*^{k}.

Clay Shade has a negative effect on both the biomass of leaves$^{(**)kk}$ and survival^{k}, but the total biomass^{k} is actually positively affected. Sulfate doesn't show any significant effects. Chloride has a negative effect on the biomass of leaves$^{(**)kk}$.

Table R3 (*Ranunculus acris*)

		leaf biom. (g)	Stdev	n	stem biom. (g)	Stdev	n	total biom. (g)	Stdev	n	survival (%)	Stdev (n=4)
sand	shade	0,045	0,066	17	0,033	0,048	17	0,074	0,112	18	72	28
	light	0,032	0,044	14	0,025	0,052	14	0,052	0,089	16	72	33
	mean diff.	0,012			0,008			0,022			0	
	sulfate	0,050	0,070	15	0,036	0,051	15	0,081	0,118	16	66	30
	no sulfate	0,028	0,040	16	0,024	0,049	16	0,048	0,084	18	78	30
	mean diff.	0,022			0,012			0,033			-13	
	chloride	0,009	0,008	4	0,008	0,006	4	0,013	0,006	7	50	23
	no chloride	0,044	0,059	27	0,033	0,052	27	0,077	0,110	27	94	7
	mean diff.	-0,035			-0,025			**-0,064^{kk}**			**-44^{k}**	
peat	shade	0,010	0,014	21	0,007	0,009	21	0,014	0,021	26	91	12
	light	0,004	0,003	9	0,003	0,001	9	0,006	0,004	10	66	26
	mean diff.	**0,005***			**0,005***			**0,008***			25	
	sulfate	0,015	0,019	9	0,010	0,013	9	0,015	0,027	15	72	12
	no sulfate	0,005	0,005	21	0,004	0,004	21	0,010	0,008	21	84	31
	mean diff.	0,010			0,005			0,006			-13	
	chloride	0,004	0,005	10	0,005	0,005	10	0,007	0,007	14	72	28
	no chloride	0,010	0,014	20	0,007	0,009	20	0,015	0,023	22	84	19
	mean diff.	-0,005			-0,002			-0,008			-13	
clay	shade	0,030	0,020	29	0,025	0,022	29	0,054	0,042	30	91	12
	light	0,069	0,049	27	0,038	0,019	27	0,106	0,065	27	94	7
	mean diff.	**-0,038$^{(***)kk}$**			**-0,012^{*kk}**			**-0,053$^{(**)kkk}$**			-3	
	sulfate	0,048	0,043	28	0,031	0,019	28	0,076	0,059	29	94	7
	no sulfate	0,050	0,041	28	0,031	0,023	28	0,082	0,061	28	91	12
	mean diff.	-0,003			-0,001			-0,006			3	
	chloride	0,031	0,029	25	0,020	0,015	25	0,052	0,041	25	88	10
	no chloride	0,063	0,045	31	0,040	0,021	31	0,100	0,063	32	97	6
	mean diff.	**-0,032$^{(**)kk}$**			**-0,020^{***kkk}**			**-0,048$^{(***)kk}$**			-9	

Manova: *p<0,05 ; **p<0,01 ; ***p< 0,001
Kruskal - Wallis: k p<0,05 ; kk p<0,01 ; kkk p< 0,001
- sign indicates that no value is available

Table R3 (*Ranunculus acris*).

Sand The effects of shade and sulfate seem slightly positive on biomasses, but not significantly. Sulfate might decrease survival, but neither this is significant. The effect of chloride, however, seems to be negative considering the biomasses of leaves, stems and the total plant^{kk}, as well as survival^{kk}.

Peat Shade has a positive effect on the biomasses of leaves*, stems* and the total plant*. There might also be a positive effect on survival. Sulfate seems to has a slightly positive effect on the biomasses, but a negative effect on survival. Chloride seems to affect both biomasses and survival negatively, but this is not significant.

Clay Shade has a negative effect on the biomasses of leaves$^{(***)kk}$, stems^{*kk}, and the total plant$^{(**)kkk}$. Sulfate doesn't seem to have any effect. Chloride has a negative effect on the biomasses of leaves$^{(**)kk}$, stems$^{***\ kkk}$, and the total plant$^{(***)kk}$.

PHOSPHATE LEVELS AND PH IN SOIL PORE WATER

Table R4 (pH and PO_4^{2-}-levels in sand and peat)

		$[PO_4^{2-}]$ (mM)	Stdev (n=3)	pH	Stdev (n=3)
sand	shade	0.166	0.205	5.577	0.425
	light	0.207	0.275	5.615	0.189
	mean diff.	-0.041		-0.038	
	sulfate	0.162	0.181	5.555	0.367
	no sulfate	0.211	0.291	5.637	0.281
	mean diff.	-0.049		-0.082	
	chloride	0.088	0.136	5.509	0.301
	no chloride	0.285	0.281	5.683	0.332
	mean diff.	**-0.198**	*k	-0.173	
peat	shade	0.412	0.400	4.203	0.297
	light	0.942	0.856	4.283	0.469
	mean diff.	-0.530		-0.080	
	sulfate	0.478	0.408	4.140	0.201
	no sulfate	0.876	0.891	4.345	0.498
	mean diff.	-0.398		-0.205	
	chloride	0.403	0.373	4.058	0.240
	no chloride	0.950	0.863	4.427	0.424
	mean diff.	**-0.547**	k	**-0.368**	**k

Table R4.
This table shows the same structure as tables 1 until 3, only here the parameters are phosphate(mM) and pH. There is no column where 'n' is mentioned, since this is a fixed number (three duplicates) for all treatments. Also in this table a negative value in the row of 'mean difference' indicates a negative effect of a treatment on the parameter. There are no measurements done in clay, since this soil type is too hard to penetrate with a rod. The same statistical tests as performed on the phytodata are performed on these data.

Chloride seems to have a negative effect on the pH of peat** ^{k}. It also decreases phosphate levels in both sand* ^{k} and peat^{k}. Neither shade nor high sulfate levels have a significant negative effect on either $[PO_4^{2-}]$ or pH.

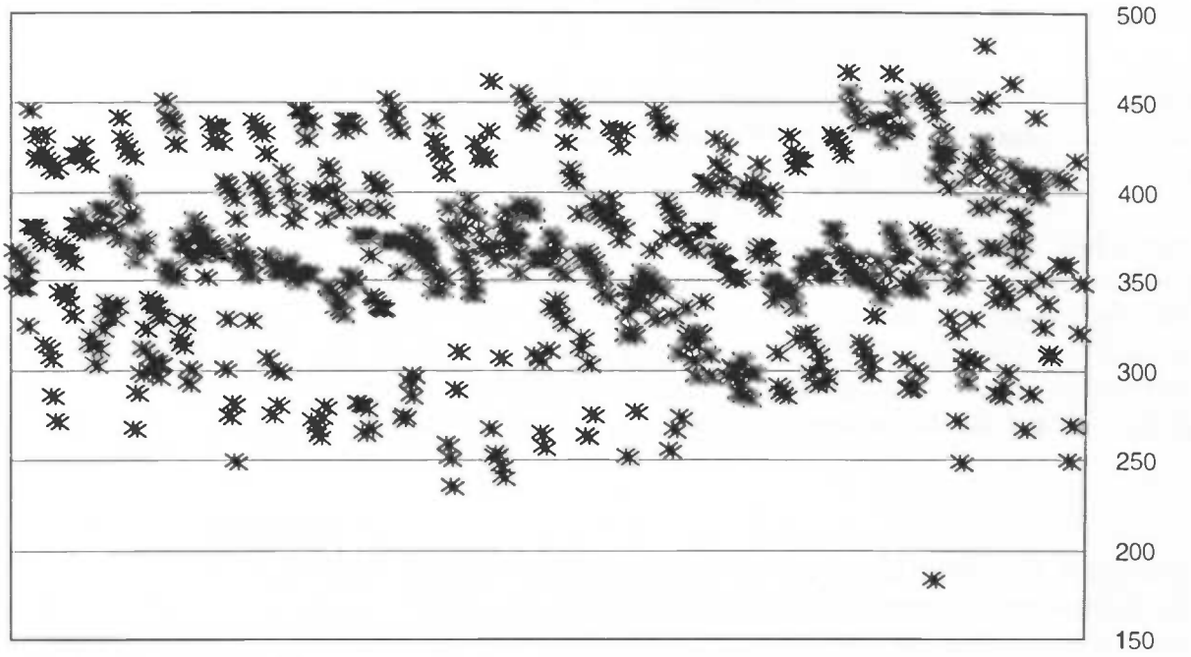


Fig. R1. Redox potential.
This graph shows the range of redox potential values found in sand and peat. It shows that the potential ranges between approximately 250 and 450. These values indicate that no reduction of sulfate into sulfide can occur. This process will only occur on a significant scale at redox potentials of <50.

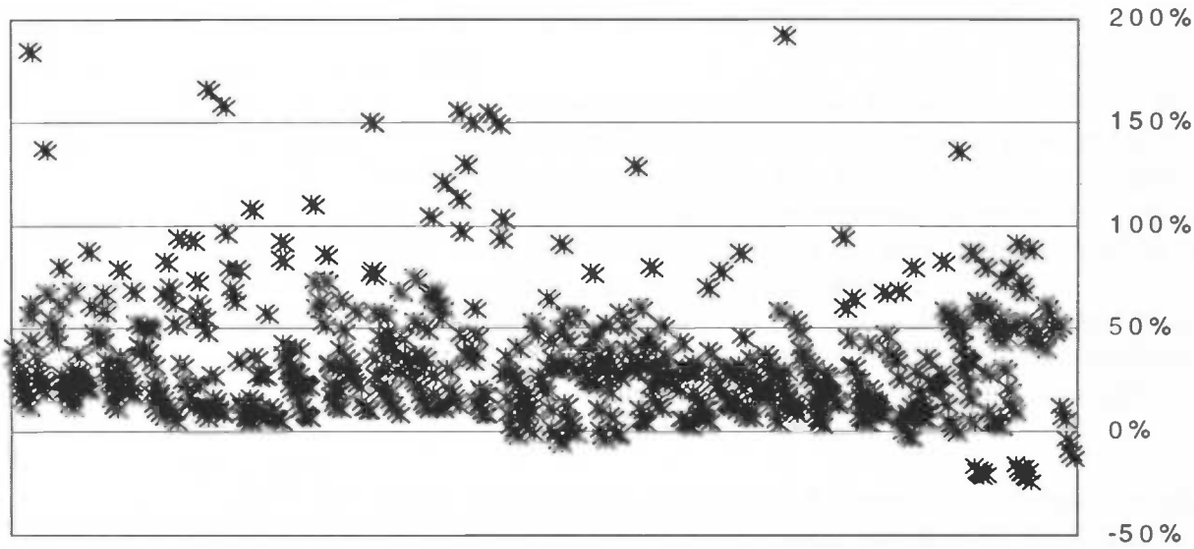


Fig. R2. Oxygen saturation.
This graph shows the range of oxygen saturation values found in sand and peat. In most soil cores there is still at least 20% - 40% oxygen saturation. No significant oxygen depletion occurred. Values above 100% show oversaturation. Values below 0% are artificial effects of the instrument

CONCLUSION/DISCUSSION

SPECIES

The results don't show that the more common species *Ranunculus acris* is less affected by the stress factors in general than the less common species *Sanguisorba officinalis* or *Caltha palustris*. For *Caltha*, Probably too little plants survived the whole business to make any significant results whatsoever, with few exceptions (table R1).
On all three species the effects of the treatments are generally quite divers; sometimes negative, sometimes positive, but most of the time just absent. From these results, it can be said that rarity of a species is not a very good predictor for capability of coping with the abiotic stress of chloride. What these results do show, is that different species (or at least *R. acris* and *S. officinalis*, who happen to differ in geographical abundance) function differently on different soils. There seems to be more effect of niche difference than of a difference in ecological range.

CALTHA PALUSTRIS (TABLE R1)

When growing on sand, *Caltha* seems to profit from high sulfate levels (table R1). According to redox measurements however (fig. R1), this can not be due to internal eutrophication via sulfide formation. There has to be another explanation. Assuming that this sulfate effect is not an artefact (considering the low number of plants measured) and considering the fact that for the other species no positive or negative effect of sulfate on biomass was found whatsoever, this effect might have something to do with properties of *Caltha palustris* itself, probably in combination with soil properties that are found in sand, but not in peat or clay. It is not known what these factors might be. It is odd, however, that *Caltha* in its natural environment is not found on sandy soils.
The lower survival of plants grown under chloride-rich conditions in clay fits well into the general effect of chloride on all three species.

SANGUISORBA OFFICINALIS (TABLE R2)

Again here, chloride and clay form a disadvantageous combination. But especially in peat, the negative effect of chloride is profoundly present. This coincides however with a low pH and a low phosphate level. This low phosphate level supports the information from literature (Schaminée 1996) that Sanguisorba grows in eutrophic environments. Conclusion is that chloride has a negative effect on *Sanguisorba* when growing on peat, possibly due to a negative effect of chloride on phosphate levels and/or pH in this soil type.
The effect of shading on the plants is difficult to summarise. It more or less shows that a plant can be affected either way by shading. Concluding these results, the differences in effects of shade are actually related to the soils and parameters, so more research is required to clarify.

RANUNCULUS ACRIS (TABLE R3)

Like the species mentioned above, biomass and survival of *R. acris* seems to be affected negatively by chloride. The stress, however, may not be caused by chloride itself, but by adjacent factors like high levels of some cations (table M1). This of course could be the case for the other two species as well.
Again the effect of shade is ambiguous. On peat it enhances biomass production, whereas on clay it lowers biomass production. On sand it shows no effect.

PARAMETERS

In this research only aboveground biomass was measured. However it would be interesting to know what happens with the root system too, since the ionic solution treatments in this experiment are expected to have a primary effect via the root system. It could make results possibly much stronger to incorporate root/shoot ratio or at least root biomass (Kotowski 2001).

OXYGEN DEPLETION

The process of oxygen depletion clearly did not occur (fig. R2), and redox potential didn't reach below 150 (fig. R1). So there was no way that sulfide would be produced either. Maybe oxygen depletion would occur only after a longer period, so it would just be a matter of time. More likely however is that the experiment was not well designed to promote oxygen depletion. The water surrounding the soil cores was refreshed by approximately a litre a day, so the water was moving. Besides, there was plenty water surface area where oxygen could diffuse. Also the watercolumn was only 9 cm deep and there was connection with the bottom of the soil cores. In a following experiment, this set-up should dramatically be altered, i.e. into a system where sulfate is not continuously added in oxygen-solution, but at once. Draining the soils, before transplanting, with sulfate-containing water could be an option. In such a system the water will come from above and sink into the soil, just as is the case in soils subject to inundation.

SULFATE, SULFIDE AND INTERNAL EUTROPHICATION

The formation of sulfide could cause plants to be stressed by sulfide-toxicity. Another possible effect could be that although sulfide is formed, this will in fact lead to internal eutrophication. In this case, phosphate that is present in the soil, but bound to cations like Iron ($Fe^{2+/3+}$) and thus not available for uptake by roots, will become available. Sulfide in its turn will then bind to those cations and lose its toxic effect (Lamers 2002, Olde Venterink 2000).
In sand and peat, sulfate did not have an effect on the levels of available phosphate (table R4), nor on aboveground biomass or survival (tables R1 until R3). From these two soils it is known that no sulfide was formed, due to oxic soils. In clay, free phosphate, oxygen saturation, redox potential or sulfide levels were not measured for practical reasons. But also in this soil, there has not been any effect of the sulfate-treatment on aboveground biomass. It is therefore likely to assume that also in this soil, no sulfate reduction occurred. It is not known, however, whether there was already sulfide present in clay, or whether internal eutrophication occurred during the experiment.
Like results were found by Olde Venterink. When rewetting soils with sulfate-enriched water, no internal eutrophication with phosphorus occurred. It was suggested that this was due to the phosphorus being present in calcium complexes, rather than in iron-complexes (Olde Venterink 2000). Another explanation could be that, because the tested clay cores were obviously quite iron-rich, sulfide would then bind to iron. This might cause internal eutrophication. However no positive effects of sulfate in clay on the plant parameters were found. These iron-sulfide complexes however may cause stress by acidification in dry soil conditions, when pyrite(FeS_2) is formed (Bronswijk 1993). This acidification by pyrite will influence nutrient solubility and availability (Golez 1997). This additional stress effect is not incorporated in this research (where conditions were always wet enough to avoid the problem) but should be taken into consideration when assessing the effects of sulfate reduction in field conditions.
What we can conclude from this outcome, is that sulfate generally doesn't have any effect on plants as long as no reduction takes place. One exception has to be mentioned: *Caltha palustris* seems to show higher biomass production when growing on sulfate-enriched sandy

soil. It is not very likely that this is due to internal eutrophication by sulfide, since redox potential didn't reach below 150. However, the tested population was very small.

Testability

The dataset obtained from this experiment was well testable referring to the plant biomasses apart from in the *Caltha* population, most plants survived well enough to provide six to eight biomass-data points per ultimate combination of treatments. This provides a population which is big enough to test with the ANOVA, necessary to show an effect of two or three factors together. Survival however could not be tested with the ANOVA, since the population subjected to one combination of factors was not duplicated. Thus survival had to be tested with a Kruskal-Wallis, and different combinations of factors had to be pooled together to make a testable population size. What's more, the Kruskal-Wallis tests were performed on the same dataset three times, which was sorted differently depending on which factor was tested for.

Transplantation-effects

Seeds of all used species have been germinated under the same circumstances. The seedlings were then transplanted into the soil cores in the experiment. A complicating factor in this approach however, is that some species could well be stressed more than others could by this action, thus *creating* a bias. Especially the low number of developing individuals of *Caltha palustris* (table R1) points in that direction.

Soils

One of the interesting aspects of this experiments is the use of soils from *in situ* the reserve. On the one hand this makes the experiment very relevant and directly applicable to the field situation. On the other hand, the used soil cores were collected in three sites and thus solely represent the soil properties of the site that they were taken from. This might create an erroneous interpretation of the conclusions when the target species will be introduced (also) on a slightly different site with slightly different conditions. Soils that were gathered from many different places however would be far from comparable. This leads to the second critical remark about soil heterogeneousity. Soils tend to be very heterogeneous in their properties, i.e. pH, on a very small scale (cm's). Even when collected from a single site, this could mean that the properties of eight soil cores of 7cm diameter differ so much from one another and within them, that it might be difficult to call those eight duplicates still 'non-differing factors'. In the worst case, your results could be a collection of outlyers. These problems could be avoided by choosing a definitely homogeneous substrate, like clean sand, potting soil or rockwool, and manipulate fertility, oxisity and so forth on a secure basis. This will however tell you less about the relevance of observed processes and plant growth in the field situation. Another option is to dramatically increase the number of duplicates, thus avoiding a great impact of small differences.

Clay

Measuring the abiotic condition of the clay cores appeared to face major practical problems. It was not possible to probe into the stiff substance with any instrument, let alone to do that 75-fold. Neither could the soil-samplers that suck out soil pore water extract more than one or two ml of water, insufficient for measurements. This, however, could be corrected for by diluting the sample.

Plant Conditions

A remark has to be made about the plants that were grown in the experiment. They all stayed very small, both in stressed and unstressed treatments. It is not known what the reason for this could be. It might have consequences for the representativity of the measurements, however, that thus were performed on marginally developed plants.

Future Research

The main imperfection of this experiment was clearly the fact that no oxygen depletion, necessary for sulfate reduction, took place. Another thing was that there was no duplicate for populations of tested plants, which makes it difficult to test for survival and impossible to test interactions for survival in this set-up. A sensible improvement in this experiment would therefore be to plant the plants in full-soil containers, without refreshed water around. This container should forth be duplicated to obtain more than one population. To avoid problems with continuous application with oxic solutions, it might be useful to load the soils in preparation for the actual experiment with high sulfate and/or high chloride levels. Another option could be to heat the solutions until anoxic in advance of application. Especially in this full-soil set-up, it might be just as practical to apply solution with a simple watering can instead of many pumps that need to be looked after, and thus are probably just as time consuming as going around with the watering can.

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APPENDICES

APPENDIX 1 (LIGHT INTENSITIES)

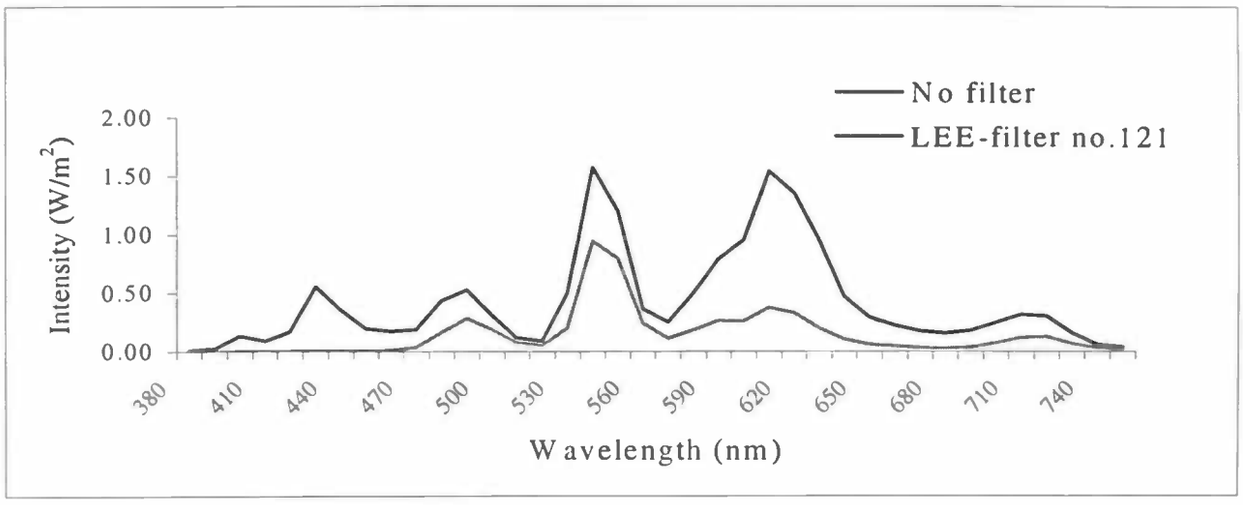


Fig. A1. light intensities
In this graph the light intensities (W/m^2) per wavelenght (nm) in the full-light treatment and the shaded situation, as measured in the climate-chambers, are shown. It shows that mainly the intensities of blue (± 445 nm) and red (± 630 nm) light are brought back by the filter. Measurements were carried out with the CENCO spectroradiometer.

APPENDIX 2 (SOIL CHARACTERISTICS)

These graphs show some of the properties of the used soils, before incorporating them into the experiment.

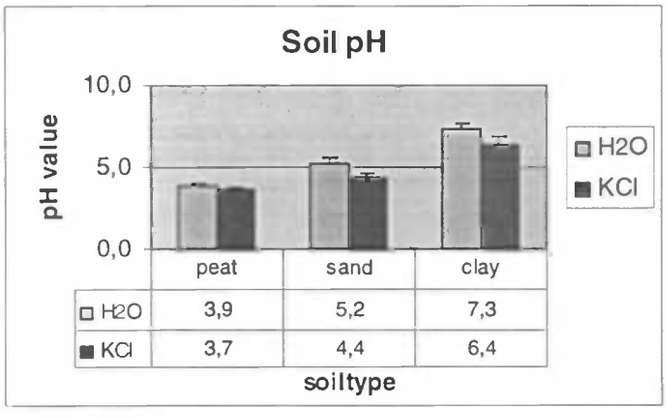
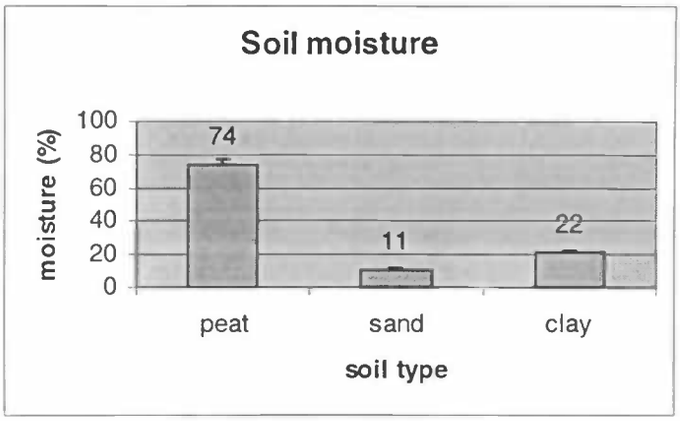
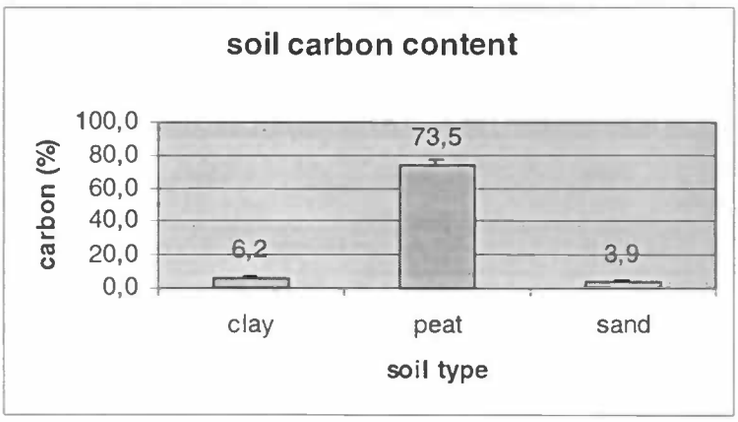


Fig. A2.1. soil pH
The soil pH was measured by two methods. The one using a water-electrode and the other using a potassium electrode. Both methods point out the same phenomenon; peat has the lowest pH and clay the highest. Sand is in-between.



Graph A2.2. soil moisture
These values were measured by drying the soils thoroughly and calculating the difference between fresh and dried soils. 'Fresh' peat consists of nearly 3/4 of water. Clay is capable to capture twice as much water as sand is.



Graph A2.3 soil carbon content
This graph shows the percentage of carbon of dried soils, which is a measure for the extent of organic matter in a soil. Understandably, peat, which is a phytogenic sediment, contains most carbon of all three considered soil types. In our case clay contains twice as much carbon/organic matter as sand does.

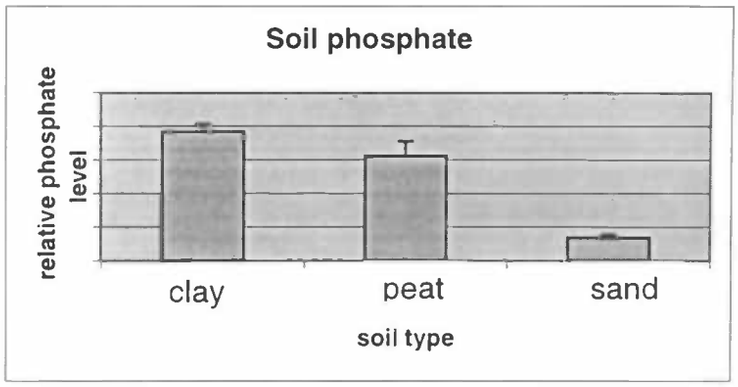


Fig. A2.4. soil phosphate
This free phosphate is measured in the same way as is done during the experiment. It shows that clay is the richest in phosphate, and sand by far the poorest.

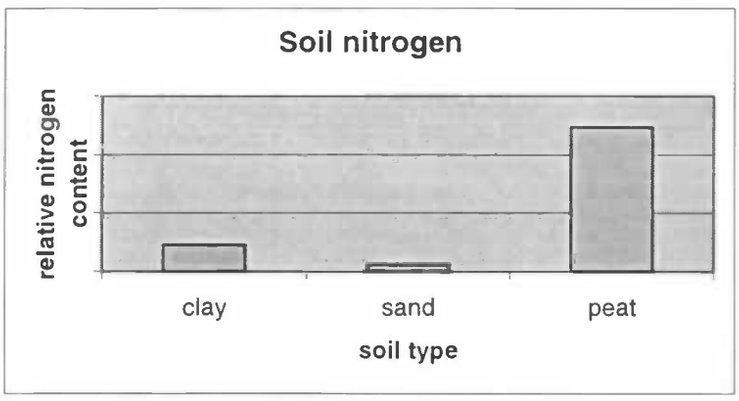
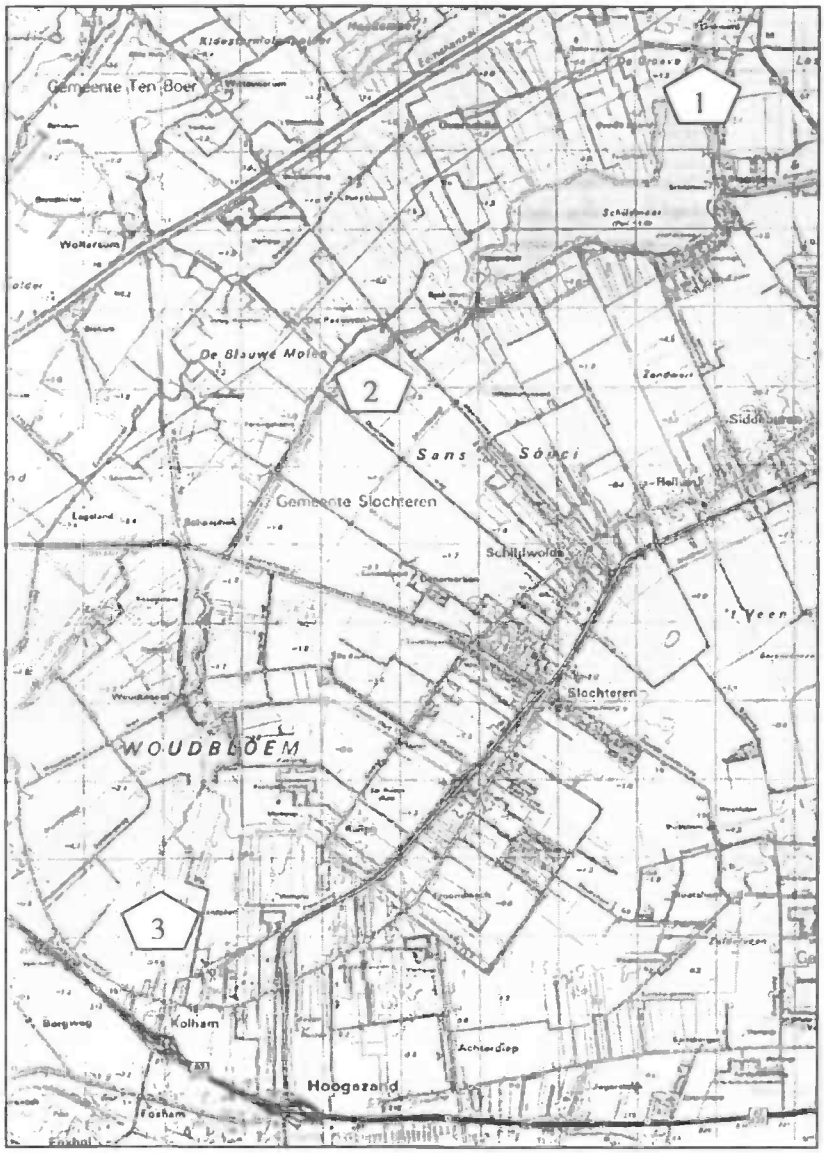


Fig. A2.5. nitrogen content
Like in soil moisture and organic matter, peat contains by far the most nitrogen of the three soils. Clay contains more nitrogen than sand.

APPENDIX 3 (ORIGINS OF SOILS USED IN THE EXPERIMENT)



Collection sites of:

1 clay

2 peat

3 sand

Fig. A3. Collection sites of tested soils from the midden Groningen area. The area of habitat creation stretches to both sides of the imaginary line between the collection sites (map from 'Topografische Dienst Nederland', leaf 7 east).