Supporting Information 2

Experimental studies to provide long-term data sets for testing population models for *Lemna* sp.

Data report *Lemna minor* microcosm monitoring study: pre-experiments to determine the appropriate nutrient load and decide if a sediment layer is necessary.

September 20, 2017

This S.I. file is related to the paper "Seasonal dynamics of the standard test species *Lemna* sp. in outdoor microcosms".

1. How the effects of nutrients are modelled in the current version of the *Lemna* model

We refer to the model of Schmitt et al. (2013). The effect of nutriens on growth is modelled via a Monod type formula using a half-saturation constant $[N]_{50}$ as the (external) concentration of the nutrient with results in 50 % inhibition of production.

$$f\,(\mathrm{N}) = \frac{[\mathrm{N}]}{[\mathrm{N}] + [\mathrm{N}]_{50}}$$

The model considers Phosporus and Nitrogen as nutrients. In the original model, the functions for both are multiplied (f(N) x f(P)) but in the Fraunhofer implementation the Min(f(N), f(P)) is used, according to Liebig's law. Thus, only the more limiting nutrient is relevant for the growth.

Note that no internal concentration and luxury consumption is modelled. The external nutrients are modelled like temperature and light, i.e. not affected by Lemna.

The half saturation constants for P and N were fitted to data from Lüönd (1983) for L. minor: **0.0043 mg P/L and 0.034 mg N/L**.

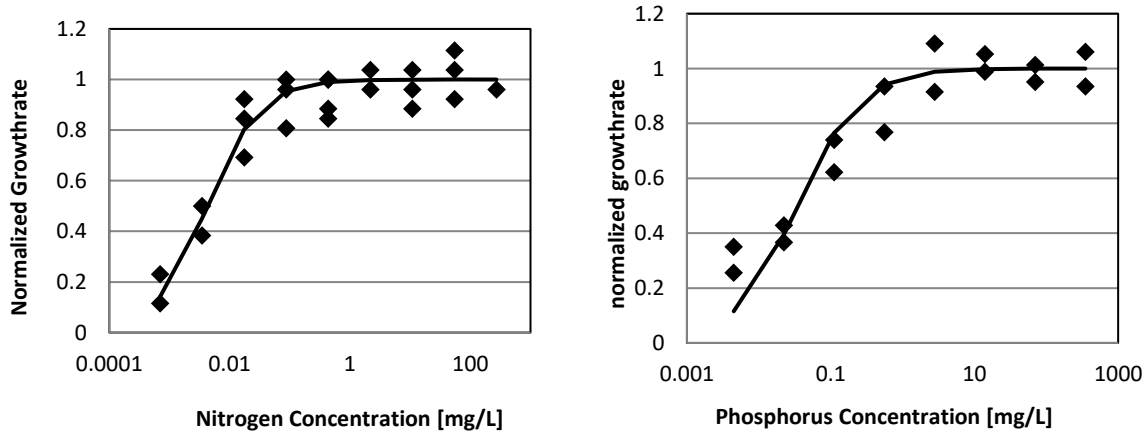


Figure 1: Growth rates of L. minor in dependence of nitrogen and phosphorus concentrations, copied from Schmitt et al. 2013, suppl. material.

Peeters et al. (2013) also developed a Lemna model but in the context of climate change research and not ecotoxicology. The growth model is very similar to the one of Schmitt et al. (2013). They used the same type of function to describe the nutrient dependency but used different half-saturation constants, interestingly based also on data of Lüönd, but from 1980: **0.05 mg P/L and 0.04 mg N/L**. The value for N is similar, but for P it is by one order of magnitude larger than the one used by Schmitt et al. (2013).

To reach 90 % of the maximum growth rate, **0.04 respectively 0.45 mg P/L** and 0.36 mg N/L would be needed according to these functions.

For comparison, the Swedish Standard (SIS) Lemna growth medium and the 20X AAP growth medium in the OECD 221 contain 2.4 or 3.7 mg P/L and 14 or 84 mg N/L, respectively. Validity criterion: growth rate > 0.275/d

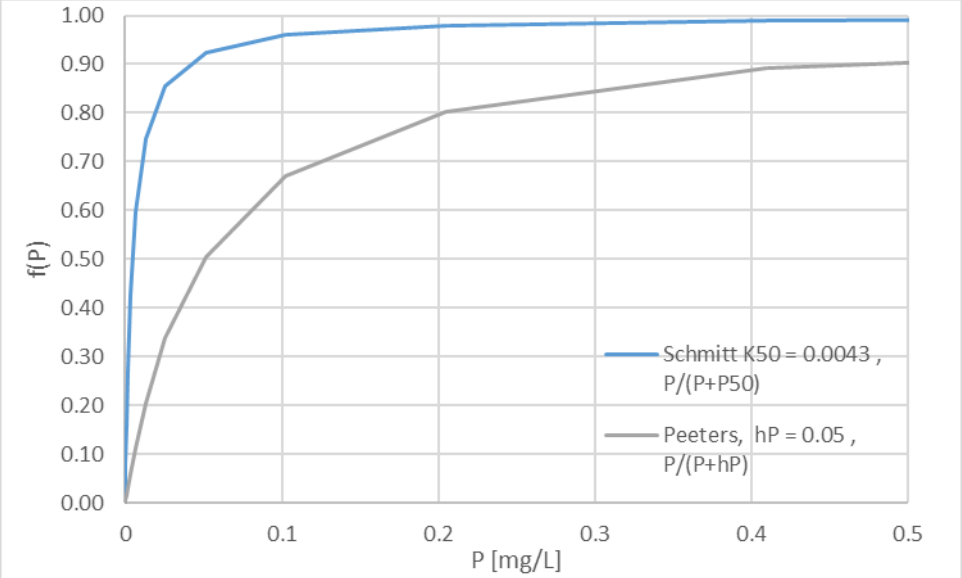


Figure 2: Growth rate as a function of phosphorus concentrations using the functions of Schmitt et al. or Peeters.

2. *Lemna* pre-experiment

In order to get insight into the nutrient range that is needed for *Lemna* growth, a *Lemna* pre-experiment was performed including the following treatments:

- Three micrococms including trays with plant soil and slow-release nutrient pellets;
- Three microcosms including trays with sediment half / half natural clay and plant soil (on a weight basis) and slow-release nutrient pellets;
- Three microcosms including trays with sediment consisting of 75 % natural clay and 25 % plant soil (on a weight basis) and slow-release nutrient pellets;

This experimental design was considered as to be preferred over a design without a sediment layer in order to ensure a slow but continuous release of nutrients from the sediment. Each microcosm was seeded with 25 *Daphnia* and 100 fronds of *Lemna* sp.. For this initial

population, individual plants were selected which had 2-3 fronds. The *Lemna* population originates from the Sinderhoeve experimental station.

The aims of this pre-experiment were:

1. Getting insight into the optimum conditions for *Lemna* growth;
2. Getting insight into the best management practice to limit algae growth in the water column;
3. Getting insight into the need for additional nutrients (K_2HPO_4 and NH_4NO_3) to be added to the water layer of the microcosms.

The pre-test was established the 12th of June and lasted for 3 weeks. The final sampling was the 3rd of July.

Results

The highest growth rate of *Lemna* was observed in the microcosms with 100 % clay. However, considering the variability between replicates, these differences were small and probably not significant (not tested). The mean growth rate was 0.061 /d for frond number and 0.081 /d for biomass (dry weight).

The type of the sediment had a larger effect on the development of algae (measured as turbidity) and, in consequence, pH and DO of the water.

At the end of the test, ammonium concentrations were higher in the 100 % peat microcosms (mean = 0.11 mg/L) than in the other microcosms (0.03 and 0.01 mg/L). The maximum phosphorus concentration found was 0.002 mg/L with an average of < 0.001 mg/L.

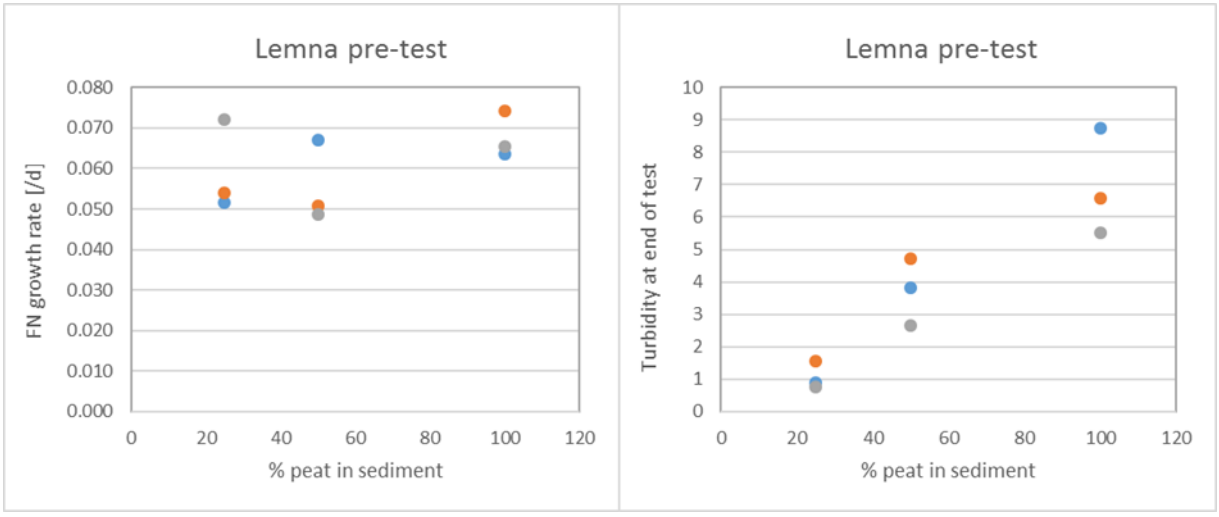


Figure 3: ***Lemna*** **pre-test: Lemna growth rate (left) and turbidity as a measure of algae abundance (right) depending on type of sediment**

Table 1: Growth rate based on number of fronds (average of three counts) and biomass (dry weigth) of Lemna minor in pre-experiment

		FN	FN	r_FN	r_bm	Turbidity	Turbidity	EC	pH	O2	Temp	N-NH4	(NO3+NO	P-PO4
Sediment		12-Jun	3-Jul			26 June	3 July				3. July ?			
peat / clay		#	#	/day	/day	?	?	µS/cm	-	mg/l	°C	[mg/l]	[mg/l]	[mg/l]
100% peat	A	100	380	0.064	0.08	7.01	8.75	214.7	10.1	13.56	19.7	0.13	5.86	-0.001
100% peat	B	100	475.33	0.074	0.10	7.33	6.57	220	10.2	13.09	19.5	0.07	6.55	0
100% peat	C	100	394.67	0.065	0.09	7.68	5.51	217.2	10.2	15.31	19.4	0.13	5.96	-0.001
50 - 50 % p	A	100	408	0.067	0.08	3.84	3.81	226	9.3	10.61	18.5	0.03	6.18	0.001
50 - 50 % p	B	100	291	0.051	0.07	3.18	4.71	224	9.3	11.17	18.4	0	6.26	-0.001
50 - 50 % p	C	100	277.67	0.049	0.07	2.16	2.67	225	9.1	10.4	18.8	0.06	6.64	0
25 - 75 % p	A	100	296	0.052	0.07	1.5	0.88	218	8.6	9.36	18.1	0.03	5.6	0.002
25 - 75 % p	B	100	310.67	0.054	0.08	1.48	1.56	213.8	8.6	10.16	18.2	0.01	5.7	0.001
25 - 75 % p	C	100	455.33	0.072	0.09	2.87	0.77	218	8.4	9.4	19	-0.01	5.53	0.001
100% peat	Mean		417	0.068	0.09	7.34	6.94	217.30	10.17	13.99	19.53	0.11	6.12	-0.001
50 - 50 % p	Mean		326	0.055	0.07	3.06	3.73	225.00	9.23	10.73	18.57	0.03	6.36	0.000
25 - 75 % p	Mean		354	0.059	0.08	1.95	1.07	216.60	8.53	9.64	18.43	0.01	5.61	0.001

Preliminary conclusions

- The type of the sediment had only a slight effect on the growth of *Lemna* over three weeks, the relative growth rate is quite similar in different treatments;
- Relative growth rates are low compared to the minimum growth rate in lab test (OECD 221);
- The data do not allow to check the type of growth, e.g. when the growth started to decline. However, it is evident that the nutrients released from the sediment was not sufficient to allow exponential growth of *Lemna* over three weeks. Probably *Lemna* would not be able to cover the water surface of the microcosms under the current conditions. Thus, nutrient solutions should be added in the main experiment.
- The introduced daphnids were apparently not able to prevent an algae bloom which was much more pronounced in the 100 % peat microcosms.
- The water column was P-limited; N is mainly available as Nitrate.

Based on these conclusions we started the *Lemna* monitoring experiment with 50- 50 % peat / clay sediment and in addition providing a low amount of extra phosphorus to the water layer in order to elevate the P-levels.

3. Lemna monitoring study

The Lemna microcosm experiment followed the experimental protocol. Data provided here are preliminary and include the sampling results up to and including 28th of August.

Phosphorus was added weekly to each microcosm. Based on a volume of 26.555 L of the water column in each microcosm, and aiming for a concentration of 0.15 mg/L P, 22,39 mg of K_2HPO_4 was added to each microcosm.

Table 2: The amount of phosphorus added to the microcosms weekly.

Target P conc mg/L	K2HPO4[mg/L]	K_2HPO_4 [mg/microcosm]	P [mol/L]
0.15	0.843	22.4	0.0048

Two microcosms were harvested on each sampling date. (Thus, we don't have frond number counts over time for the same microcosms and calculated growth rates cannot be based on measurements in the same microcosm).

Results

Lemna grew only over the first 11 days. Later in the study, only one microcosm (No. 23) showed a high number of fronds (1360 compared to upt to ca. 400 in the other microcosms).

Over the first 11 days that growth rate was 0.12/d but from Day 11 to Day 40 the growth rates were very low. If we assume that in microcosm 23 the frond number on Day 39 was equal to the mean of the number measured in two other microcosms, the growht rate for microcosm 23 would be 0.22. However, it might be that the frond number in microcosm 23 was already higher than in the other microcosms.

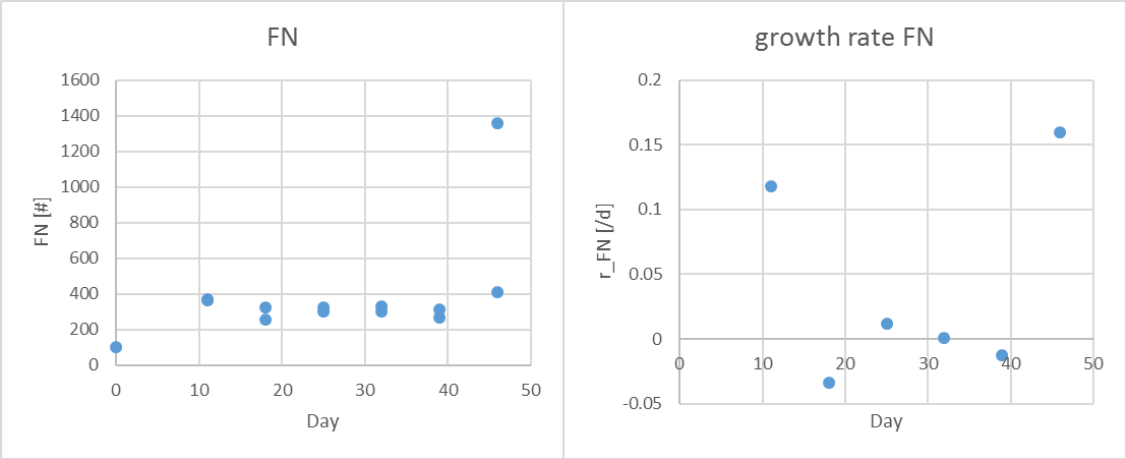


Figure 4: Lemna main test results until 28. Aug 2017: Lemna frond number (left, data from 2 replicates) and growth rate (right, means of the two replicates) over time

Table 3: Results of Lemna monitoring study up to and including 28th of augustus
The growth rates for frond number were recalculated per week instead for the whole test duration (as for biomass)

Date	Day nr.	Cosm No	FN #	FN mean	FN r /d	BM r /d	EC µS/cm	pH	O2 mg/L	Temp	Turbidity	N-NH4 [mg/l]	NO3+NO [mg/l]	P-PO4 [mg/l]
13. Jul	0	start	100	100			?	?	?	?	?			
24. Jul	11	18	366			0.11	201.8	10.05	9.8	12.72	18.1			
24. Jul	11	42	369	367.5	0.11832	0.11	207.2	9.7	13.11	18.3	3.5			
31. Jul	18	34	258			0.08	227	10.1	12.52	17.3	5.23	0.02	3.52	0.000
31. Jul	18	66	322	290	-0.03383	0.08	214.4	9.4	10	176	3.19	0.10	4.08	0.030
07. Aug	25	11	327			0.06	197.8	9.1	10.35	18	3.12	0.09	2.84	0.003
07. Aug	25	63	302	314.5	0.01159	0.05	227	10	14.39	17.2	12.15	0.02	3.83	0.001
14. Aug	32	33	303			0.04	215	9.5	11.43	17.5		0.05	2.85	0.001
14. Aug	32	61	329	316	0.00068	0.05	221	8.5	8.55	17.7		0.21	5.08	0.011
21. Aug	39	3	312			0.02	223	9.6	12.75	16.6				
21. Aug	39	45	268	290	-0.01227	0.04	208.4	9.6	9.59	16.6				
28. Aug	46	23	1360				202.4	9	10.98	18.5				
28. Aug	46	67	412	886	0.15955		207.3	9.3	11.17	18.3				

Recovery tests

Every week, the 2 harversted microcosms were re-inoculated with 100 Lemna fronds to measure growth rates without relevant intraspecific competition.

The test should provide a data base to analyse growth rate under outdoor conditions as a function of temperature and light (under the assumption of no nutrient limitation..).

Growth rates were between 0.05 and 0.08 /d over a period of 14 days but above 0.15 /d in the last test over 7 days.

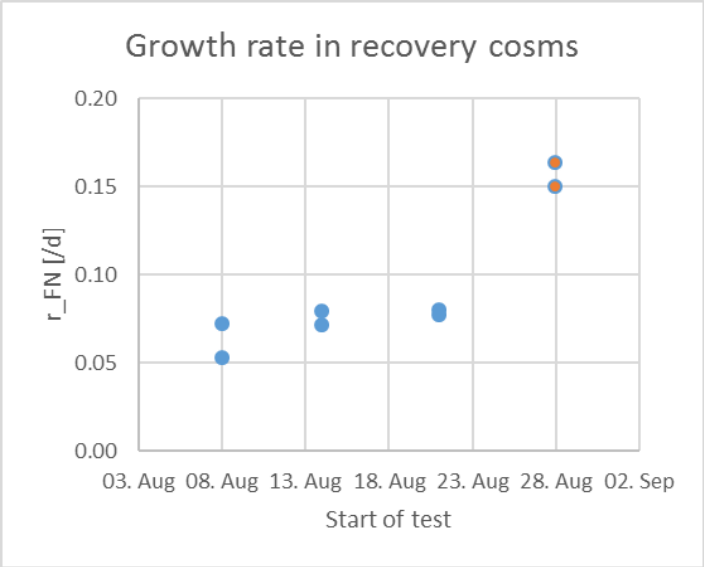


Figure 5: Lemna growth rates in the recovery tests. Note that the first three test lasted 14 days while the last test lasted 7 days.

Table 4: Results of recovery of Lemna in Lemna monitoring study

Date	Days	Microcosm	Recovery	Growth	Relative Growth rate	Relative Growth rate	
		nr.	average N fronds	%	N fronds Day-1	Biomass Day-1	
8-aug	14	18	210	110	0.05	0.08	
8-aug	14	42	275	175	0.07	0.10	
14-aug	14	34	304	204	0.08	0.09	
14-aug	14	66	273	173	0.07	0.09	
21-aug	14	11	296	196	0.08	0.10	
21-aug	14	63	308	208	0.08	0.09	
28-aug	7*	3	314	214	0.16		
28-aug	7*	45	285	185	0.15		

*Incorrectly sampled after 7 d instead of 14 d

Table 5: Water quality parameters in Lemna minor microcosms

Date	Day nr	Microcosm	EC	pH	O2	Temp	Turbidity
			microS/cm		mg/L		
13-jul	0	start					
24-jul	11	18	201.8	10.05	9.8	17.72	18.1
24-jul	11	42	207.2	9.7	13.11	18.3	3.5
31-jul	18	34	227	10.1	12.52	17.3	5.23
31-jul	18	66	214.4	9.4	10	176	3.19
7-aug	25	11	197.8	9.1	10.35	18	3.12
7-aug	25	63	227	10	14.39	17.2	12.15
14-aug	32	33	215	9.5	11.43	17.5	
14-aug	32	61	221	8.5	8.55	17.7	
21-aug	39	3	223	9.6	12.75	16.6	
21-aug	39	45	208.4	9.6	9.59	16.6	
28-aug	46	23	202.4	9	10.98	18.5	
28-aug	46	67	207.3	9.3	11.17	18.3	

Table 6: Water quality parameters in Lemna minor recovery microcosms measured at the end of the recovery tests

Date	Day nr	Microcosm	EC	pH	O2	Temp	
			microS/cm		mg/L		
24-jul	0	start					
8-aug	11	18	218.7	9.7	10.96	18.2	
8-aug	11	42	244	10.5	18.76	17.7	
14-aug	18	34	226	9.7	14.59	18.1	
14-aug	18	66	219.5	9.9	12.8	18	
21-aug	25	11	185	8.1	10.92	17.6	
21-aug	25	63	211.8	8.7	8.24	17.3	
28-aug	32	33	208.2	9.5	11.59	16.5	
28-aug	32	61	213.8	8.8	9.78	16.6	
4-sept	39	3	226	9.3	9.33	18	
4-sept	39	45	217.9	9	10.04	18.1	

Table 7: Nutrient concentrations in Lemna minor microcosms.

Lab nr.	**Monster-omschrijving**	**N-NH4 [mg/l]**	**N-(NO3+NO2) [mg/l]**	**P-PO4 [mg/l]**
	detection limit	0.04	0.03	0.02
data 17 AUG				
1	microcosm- 11	0.09	2.84	0.003
2	microcosm- 33	0.05	2.85	0.001
3	microcosm- 34	0.02	3.52	0.000
4	microcosm- 61	0.21	5.08	0.011
5	microcosm- 63	0.02	3.83	0.001
6	microcosm- 66	0.10	4.08	0.030
Lab nr.	**Monster-omschrijving**	**N-NH4 [mg/l]**	**N-(NO3+NO2) [mg/l]**	**P-PO4 [mg/l]**

	detection limit	0.04	0.03	0.02
data 29 June				
1	25 % peat 75 % clay	0.03	5.60	0.002
2	25 % peat 75 % clay	0.01	5.70	0.001
3	25 % peat 75 % clay	-0.01	5.53	0.001
4	50 % peat 50 % clay	0.03	6.18	0.001
5	50 % peat 50 % clay	0.00	6.26	-0.001
6	50 % peat 50 % clay	0.06	6.64	0.000
7	100 % peat	0.13	5.86	-0.001
8	100 % peat	0.07	6.55	0.000
9	100 % peat	0.13	5.96	-0.001
10	Myr ditch 13 - 10 meter	0.03	0.02	0.001
11	Myr ditch13 - 20 meter	0.03	0.00	0.001
12	Myr ditch 13 - 30 meter	0.03	0.00	0.001

Preliminary conclusions

- Relative growth rates seem to decrease over time, based on the observation that the relative growt rates are higher in the recovery studies and in the first week of the experiment;
- In general, growth rates are low;
- Turbidity is variable, which corresponds to the variability in colour (some are greener than others); but also *Lemna* growth is variable (see microcosm 23)
- P is still very low; the water column may be still P-limited;
- N is mainly available as Nitrate;
- The N/P ratios are unfavourable in the microcosms. This might be the issue here. The Sinderhoeve groundwater naturally has much higher nitrate concentrations than phosphorus concentrations. A ratio of 6 is favourable for Lemna growth (P 6x N based on mol weight);
- Microcosms show high variability in *Lemna* growth.

We advice the following options to continue:

- We advice to increase P-levels or alternatetively increase P-levels after the *Lemna* layer has reached full coverage of the water surface (which is the case in one microcosm now) in order to limit competition by algae; based on literature data we suggest to add P with a target concentration of at least 0.5 mg/L, which is about three times higher than the current weekly loadings.

Target P conc mg/L	**K2HPO4[mg/L]**	**K_2HPO_4 [mg/microcosm]**	**P [mol/L]**
0.5	2.81	74.6	0.016

- The systems seem to show some (typical) but relevant variability. Time series of frond numbers measured always in other microcosms might be misleading because it is not clear whether the values of the previous sampling are representative for these microcosms. Therefore, we advice to sample the recovery experiments after 7 instead of 14 days to get a better estimation of the potential growth. Over 14 days, growth seems to be more limited. With this greater frequency of recovery, we will focus on FN, with measurement of biomass less frequently.

- Given the high variability among the microcosms (which turned out to be higher than expected), we advice to monitor the same microcosms over time (but then only FN) and used harvested microcosms only to measure BM / FN. A set of e.g. 5 microcosms could be monitored over the full season.

- We advice to continue sampling of the microcosms, which still have *Lemna* in it (some are empty due to unknown reasons) and in which *Lemna* shows growth. We will start the winter with all microcosms having a full cover of *Lemna* by re-introduction of new *Lemna* fronds and study overwintering and re-growth in spring.

- A possibility is to use *Lemna* fronds of mother populations grown up in nutrient-rich water; these *Lemna* fronds have accumulated nutrients due to luxury consumption and can use these nutients in the first period of the experiment (See paper of Peeters et al. (2016)). Also Coors et al. (2006) found that bioassays in mesocosms showed higher control growth rates when the *Lemna* fronds were taken from lab culture instead from other mesocosms.

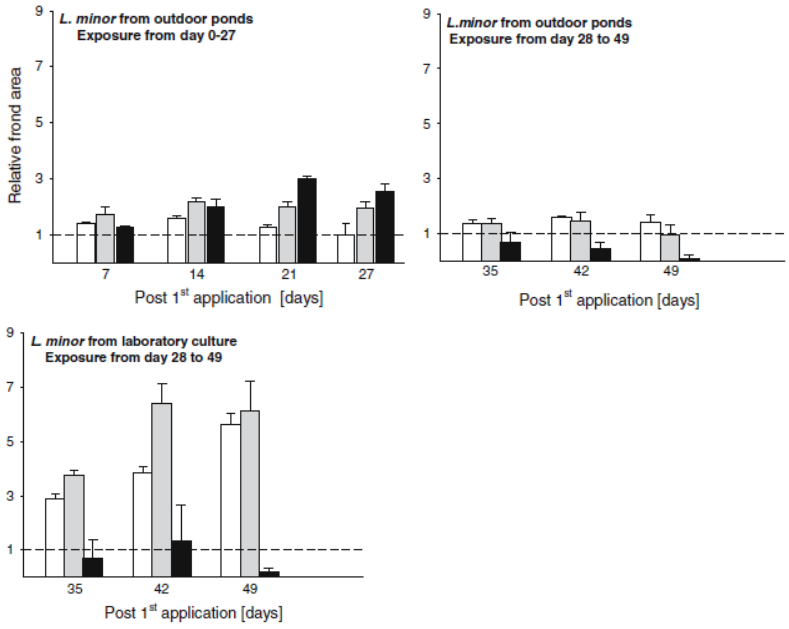


Figure 5: Effects on the frond area of L. minor in in-situ bioassays (copy from Coors et al., 2006).

However, luxury consumption is not included in the model yet… We don't know how long the internal storage will allow growth. Thus, adding nutrients to the microcosms is still needed.

References

Coors, A., Kuckelkorn, J., Hammers-Wirtz, M., Strauss, T., 2006. Application of in-situ bioassays with macrophytes in aquatic mesocosm studies. Ecotoxicology, 15 (7), pp. 583-591.

Driever, S.M., van Nes, E.H., Roijackers, R.M.M., 2005. Growth limitation of Lemna minor due to high plant density. Aquatic Botany 81, 245–251.

Lüönd, A. (1980) Effects of nitrogen and phosphorus upon the growth of some Lemnaceae. Biosystematic Investigations in the Family of Duckweeds (Lemnaceae), Vol. 1. (ed. E. Landolt), pp. 118–141, Veröffentlichungen des Geobotanischen Institutes der ETH, Zürich.

Lüönd A. (1983) Das Wachstum von Wasserlinsen in Abhängigkeit des Nährstoffangebots insbesondere Phosphor und Stickstoff in Biosystematic investigations in the family of duckweeds. Vol 3, Dissertation ETH Zürich, 1983

Peeters, E. T. H. M., van Zuidam, J. P., van Zuidam, B. G., Van Nes, E. H., Kosten, S., Heuts, P. G. M., Roijackers, R. M. M., Netten, J. J. C. and Scheffer, M. (2013), Changing weather conditions and floating plants in temperate drainage ditches. J Appl Ecol, 50: 585–593. doi:10.1111/1365-2664.12066

Peeters, E.T.H.M., Neefjes, R.E.M., Van Zuidam, B.G., 2016. Competition between free-floating plants is strongly driven by previously experienced phosphorus concentrations in the water column. PLoS ONE, 11 (9), art. no. e0162780.

Portielje, R., Roijackers, R.M.M., 1995. Primary succession of aquatic macrophytes in experimental ditches in relation to nutrient input. Aquatic Botany, 50 (2), pp. 127-140.

Schmitt W, Bruns E, Dollinger M, Sowig P (2013) Mechanistic TK/TD-model simulating the effect of growth inhibitors on *Lemna* populations. Ecological Modelling 255, 1-10

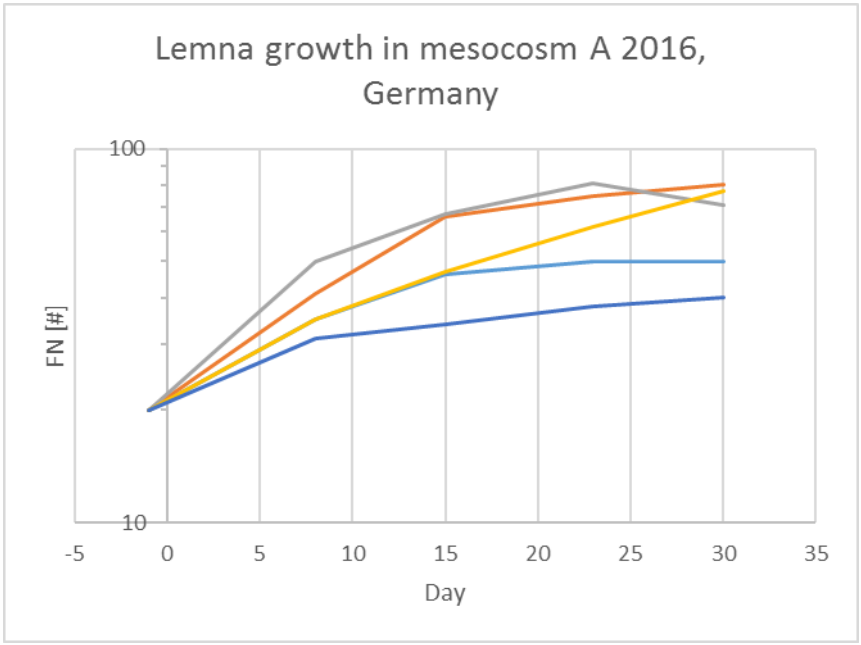
Appendix 1: Experience in other mesocosm studies

Lemna gibba in 5 control enclosures including sediment in Germany, Day 0 = 7. July 2016

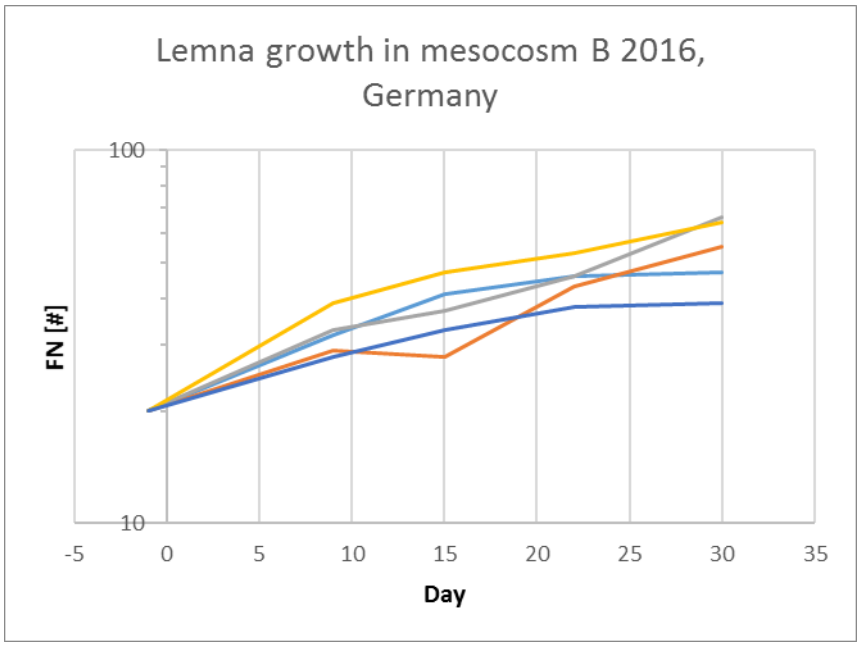
Phosphate = 0.2 mg/L on Day -1 and Day 30(**0.065 mg P/L**).

Nitrate < 0.01 mg/, Ammonium < 0.01 mg/L

Growth rate over the first 9 days reach from **0.05 – 0.1 /d**



In another mesocosm at the same test site but three weeks earlier, the initial growth was lower (0.03 – 0.07) while the P-level were higher (ca. 0.1 mg P/L). (Weather data were not checked.)



Nutrient levels in edge of field water bodies

Driever et al. 2005: Measurements in three ditches around Wageningen in summer 2003: 0.4 – 1.6 mg P/L!

Table 1
Characteristics of the ditches

Characteristic	Sinderhoeve	Zetten	Opheusden
Length (m)	40.0	45.5	48.0
Width (m)	3.0	1.8–3.0	0.7
Depth (m)	1.6	0.5	0.4
Sediment	Sand	Clay	Clay
$N–NH_4^+$ (mg l^{-1})	0.07 (±0.03)	0.08 (±0.01)	0.14(±0.10)
$N–NO_3^-$ (mg l^{-1})	0.05 (±0.05)	0.04 (±0.03)	0.99(±1.38)
$P–PO_4^{3-}$ (mg l^{-1})	0.42(±0.56)	0.51 (±0.68)	1.62 (±0.65)

The nutrient levels are mean values for the last three sampling dates.

Peeters et al. (2013): '*Fourth, the effect of nutrient limitation was evaluated using a different set of field observations from 42 ditches in the periods 26 June–17 July and 14 September–5 October in 2007 (J.P. Van Zuidam & E.T.H.M. Peeters, unpublished data) further referred to as the duckweed–nutrient data set. In both periods, duckweed biomasses (wet weight) were determined; however, orthophosphate, ammonium and nitrate were only measured in September–October. …(Simulation results): With nutrient limitation, duckweed dominance occurred later in the year than without nutrient limitation. Orthophosphate concentrations below 0.05 mg PO4 /L did not allow for duckweed dominance in any scenario. … For* ***(current) nutrient concentrations of 0.50 mg PO4 /L****, a reduction from 40% (G scenario) up to 60% (Wplus scenario) is needed to compensate for warming effects* '

Portielje et al. (1995) showed that *Lemna* could only reach full coverage of the water layer if the storage capacity of nutrients in sediment and macrophytes was exceeded by the nutrient loading. Only then nutrient concentrations permanantly increase in the waterlayer to enable *Lemna* growth.