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Effects of a Pesticide Mixture on Plankton in Freshwater Mesocosms – from single substance studies to combination impacts

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Abbreviations

a.i.	active ingredient
approx.	approximately
a.t.	after treatment; "negative " days mean "before treatment"
CA	concentration addition; a model for the prediction of a mixture toxicity (for agents with a similar mode of action)
CCA	Canonical correspondence analysis; multivariate factor analysis that combined releveé and environmental data
CYP	α-Cypermethrin, a neurotoxic pyrethriod insecticide
CYP<number>	Treatment level; the name of an enclosure containing a certain amount of α-Cypermethrin; higher numbers indicate higher concentrations
df	degrees of freedom
DT_{50}	Dissipation time for half the amount of a substance; half-life time
e.g.	for example
EAC	Ecologically acceptable concentration; the concentration of an agent that can be accepted in the environment, because its effects can be compensated in a very short time
$EC_{<number, x>}$	Effect concentration; the number means the percentage of the endpoint that show a certain effect, "x" is a wild card
et al.	and other co-authors
etc.	et cetera
GC-ECD	Gas chromatography with an Electrode Array Detection
HPLC	High pressure/performance liquid chromatography
i.e.	id est, Latin for "that means"
IA	independent action; a model for the prediction of a mixture toxicity (for agents with a dissimilar mode of action)
IPQ	Index of prediction quality; a number that indicates the accuracy of a prediction model
IPU	Isoproturon, a phenylurea herbicide, blocks photosynthesis
IPU<number>	Treatment level; the name of an enclosure containing a certain amount of Isoproturon; higher numbers indicate higher concentrations
LC	liquid chromatography
$LC_{<number, x>}$	Lethal concentration; the number means the percentage of the endpoint that were killed, "x" is a wild card
$LD_{<number, x>}$	lethal dose; the amount of an agent that kills the percentage of a test organism indicated by the number; "x" is a wild card
level <number>	mean value of a parameter in the enclosures that received a certain amount of both pesticides; higher numbers indicate higher concentrations
LOEC	Lowest observed effect concentration; the lowest treatment level at which statistically significant effects were observed
LOEL	Lowest observed effect level; the lowest treatment level at which effects were observed
LOD	limit of detection
LOQ	limit of quantification
NEC	No effect concentration; the concentration of an agent at which no effect is expected, needs not to be realized in an experiment but is retrieved by inverse regression, for example
NOAEL	No observed adverse effect level
NOEAEC	No Observed Ecologically Adverse Effect Concentration
NOEC	No observed effect concentration; the treatment level at which no statistically significant effects were observed
NOEL	No observed effect level; the treatment level at which no effects were observed, in this thesis derived by combining several results
PCA	Principal component analysis; a multivariate analysis
PE	polyethylene
PEC	peak environmental concentrations
PRC	Principal response curve; a multivariate analysis designed to test and display treatment effects that change across time
RAD	Relative absolute distance
SPE	solid phase extraction
std. dev.	standard deviation
TER	toxicity exposure ratio
UV/VIS	Ultraviolett/visible light

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1 Introduction

1.1 Plant protection and the environment

In agriculture, the use of plant protection products is a common feature. Unfortunately, even if properly used, these products do not stay where they have been applied, i.e. in the field, but are transported to near-by compartments of the environment (e.g. KREUGER and TÖRNQVIST 1998, HÖCKER and NEGELE unpublished, HOUSE *et al.* 1997, GARAMOUMA *et al.* 1998, NEAL *et al.* 2000). Since these products have been designed to impose negative impact on some organisms, other unwelcome effects to so-called "non-target" organisms (i.e. organisms that are not intended to be affected by the agent) may occur. Therefore, the use of plant protection products is inevitably a potential risk to the environment. To be more specific, the active ingredients (a.i.) of these products are concerned. In order to minimize environmental risks, agents that are to be placed on the market in the EU must be authorized. Guidance for authorization is given in the Council Directive 91/414/EEC and its amendments (EU 1991). If an active ingredient does not meet the requirements established there, it cannot (legally) be sold and used. For this reason, assessing the potential risk of plant protection agents to the environment has become a common feature by now. According to EU 2002 there are two prerequisites for environmental risk assessment:

1. Definition of suitable assessment endpoints which are understood as formal expressions of the environmental values to be protected;
2. Establishment of a certain level of protection which encompasses the acceptability of effects and the uncertainty linked to the prediction of effects.

As a general conclusion, the sustainability of populations of non-target organisms is to be protected. Since small ponds, lakes, or brooks can often be found in an agricultural landscape and because plant protection agents readily enter a water body (due to run-off after rainfall events, improper use when cleaning equipment on a farm and so on, e.g. BEERNAERTS *et al.* 1999, BBA 1997, NITSCHKE and SCHÜSSLER 1998), special interest is put in the aquatic environment. Not the least reason is that some active ingredients of plant protection products have already been found in drinking water (KASTENBERG and YEH 1993, LOEWY *et al.* 2003).

In order to achieve the aims mentioned above, a so-called "tiered" approach is proposed (EU 2002, simplified): First of all, some standard toxicological tests in the laboratory with e.g. *Daphnia magna* (Cladocera, crutsaceae, DIN EN ISO 6341) or *Scenedesmus subspicatus* (green algae, DIN EN 28 692) have to be conducted. Then the so-called "peak environmental concentrations" (PEC) have to be calculated; i.e. concentrations that can be expected to be found in water bodies near a field. In Germany, the model of GANZELMEIER *et al.* 1995 is recommended. Together with the data produced in the laboratory tests, the "toxicity exposure ratio" (TER) can be derived. TER values <100 or <10, depending on the organism and the exposure regime, trigger the requirement of multi-species tests, such as outdoor mesocosm experiments. Guidance for such tests is given in EU 2002, CLASSIC 2001, or HARAP 1999. The general concept is to implement some untreated controls and to compare the effects of at least three treatment levels with these controls on each sampling date. By doing so, the percentage of individuals of a species/population/aggregation that shows an effect can be calculated; a

regular EC_x. The controls enable researchers to estimate the natural variability of the model ecosystem. When an endpoint in a treated pond reaches the control range again (mostly twice or thrice the standard deviation is chosen as a threshold value), the ecosystem is said to have "recovered" from the effect of the pesticide. Further details on such test designs is given in EU 2002, MAISE 2002, or HARAP 1999.

By such studies, ecotoxicological endpoints like a NOEAEC (No Observed Ecologically Adverse Effect Concentration) can be derived, either with or without an additional safety factor, depending on the quality of the study (EU 2002). A mesocosm is a surrogate for a "real" ecosystem and is intended to simulate the effects a stressor will have on whole communities in a natural water body. Normally it is not connected to any other water body, so repopulation after a treatment may be hindered. In this way, a mesocosm experiment is believed to be a "worst-case" scenario (CLASSIC 2001). In any case, a mesocosm is supposed to be self-sustaining and therefore should include all kinds of functional groups of a natural ecosystem from primary producers to predators (excluding fish, EU 2002).

For each level of aggregation (species to populations) the time for recovery can be measured with such a mesocosm study. It is therefore advised to assess effects at least for 2-3 generation times of susceptible organisms (CLASSIC 2001). Of course, this recovery time will depend on the half-life of the active ingredient. Effects that last longer than 8 weeks are believed to be problematical and therefore no NOEAEC can be noted (EU 2002).

Additionally, other endpoints like an EC_x (Effect Concentration) or the NOEC (No Observed Effect Concentration) of arbitrary taxa/populations/communities may be estimated (MAISE 2002, CLASSIC 2001).

In short, a lot of parameters regarding the environmental safety of active ingredients of plant protection agents have to be collected. If an active ingredient is able to pass all these tests and if it is used properly, environmental risk can be minimized.

1.2 Environment and plant protection

Imagine two farmers whose fields are near one small pond. Let's assume both of them grow the same crop on these fields. Consequently, both may experience the same problems they want to solve with a plant protection product. By chance, the first farmer buys a product A and the second farmer buys a product B which is equally effective as product A, but has a different active ingredient (a.i.). Under these circumstances these two different a.i. on the market can be enough that both of them can be found in the near-by pond. Moreover, combining different pesticides is even proposed in recommendations of agriculture (e.g. BAYWA Agrar 2000). In this way, a scenario where some a.i. are found together in a natural water body is the rule and not the exception. In 1978 BUTLER already pointed out that more attention should be paid to the differences in the effects of a combination of stressors in contrast to the single substances.

In 1.1, the outline of the precautions have been noted that have to be taken for a specific a.i. for the environment. When looking at the results of pesticide analyses from natural waters, mixtures of pesticides have commonly been found in a lot of different studies throughout the years (e.g. GILLIOM *et al.* 1999, KREUGER and TÖRNQVIST 1998, HÖCKER and NEGELE

unpublished, HOUSE *et al.* 1997, GARAMOUMA *et al.* 1998, NEAL *et al.* 2000). Contrastingly, risk assessment is merely conducted for single substances.

This is quite surprising, because risk assessment principles like the determination of an EC_x, a NOEC or a EAC (Environmentally Acceptably Concentration) can all be applied to mixtures as well. Indeed, it is an everyday experience with medical products that at least interactions of widely used drugs are described in the instruction leaflets. None of this is the case for the "drugs" of our agriculture.

One reason for this may be the great number of plant protection agents on the market. In Germany alone about 954 different plant protection products are authorized (BVL 2003). Because testing every possible combination is therefore absolutely impossible, the question arises where to begin searching for mixture toxicity.

In any case, the actions that are taken to protect the environment from undesired effects of plant protection products may be insufficient. Till now, mixture toxicity is totally neglected in the authorization process either because of the difficulties that arise when testing the impact of mere single substances or because of gaps in our knowledge how to approach the problems met with such mixtures scientifically.

1.3 Measuring mixture toxicity: Implications and approaches

As mentioned above, testing all different kinds of mixtures is simply impossible because of the great number of combinations. Therefore, methods must be applied that can estimate the impact of a mixture by using the data generated in single substance experiments.

The first approach is to predict the mixture toxicity by looking at the active ingredients themselves instead of their effects on certain endpoints. This is done by QSAR analyses (Quantitative Structure-Activity Relationship). Certain properties of the toxic molecules are evaluated (like the hydrophobicity, certain binding sites, chain groups etc.). By common molecular characteristics a mixture toxicity is predictable (MARCHINI *et al.* 1999, YU *et al.* 2001, LIN *et al.* 2002).

Secondly, data of single species tests (or similar studies) can be used to calculate the effects of a mixture. Two different models are available: Concentration Addition (CA) (LOEWE 1927 in WALTER 2002) and Independent Action (IA, BLISS independence, response addition) (BLISS 1939). Both concepts are wide-spread and have already proven their ability of predicting combination effects (c.f. e.g. VIGHI *et al.* 2003, BACKHAUS *et al.* 2000, GRIMME *et al.* 2000, BACKHAUS *et al.* 2000a, ALTENBURGER *et al.* 2000, CLEUVERS 2002, FAUST *et al.* 2003, CLEUVERS 2003). The major difference is the mode of action that the two approaches imply. Both of them are able to use EC_x and concentration data from studies conducted in the past so the mixture toxicity for (m)any combination(s) of substances may be calculated without further research (provided that the data has been generated and documented properly). In the following, discussion will be limited to these two approaches.

Starting with CA, mere addition of the concentrations causing an effect is the basic concept behind the model. The different agents in a mixture are believed to be fully exchangeable. In other words, using 50 µg of substance A and B together (100 µg applied in total) will cause the same effects as applying 100 µg of substance A *or* B. This concept is valid if substances with a similar mode of action on a molecular basis are used.

The IA rather adds up the effects of a treatment. Therefore it is also called "response addition". The algorithm implies that when each substance (A or B) have an effect level of 50%, A *and* B will affect 75%, for example. In other words, if agent A kills 50% of a population, agent B can only kill 50% of the remaining individuals. The effect for the whole initial population will be a loss of 75%. This concept works fine for substances with a dissimilar mode of action, again on a molecular basis.

A major problem for the prediction is the occurrence of synergetic or antagonistic effects in a combination (ALTENBURGER *et al.* 1990, BLISS 1939, LUTZ *et al.* 2002, TANAKA *et al.* 2002, GRECO *et al.* 1995). For instance, it is commonly known that you should not enjoy an alcoholic drink together with certain mushrooms (unless you have suicidal tendencies). How to determine if such a pattern exists is described in ALTENBURGER *et al.* 1990, for example (by so-called isobolograms). In other words: one substance alone may not cause any effect at all but together with another problems may occur. This has been recognized by some researchers (WALTER 2002, FAUST *et al.* 2003). As a consequence, the concept of a NOEC is being heavily discussed (e.g. WALTER 2002, FAUST *et al.* 2003, BACKHAUS *et al.* 2000, FAUST *et al.* 2001, HANSON and SOLOMON 2002). For single species tests there are some hints that a NOEC is indeed not as "safe" as it is intended to be. Even for substances with a different mode of action on a molecular basis synergistic effects have been reported in laboratory single species tests (WALTER 2002).

Combination effects may be triggered indirectly and therefore cannot easily be estimated in advance. This especially holds true for secondary reactions in an ecosystem. Suppose you investigate the mixture toxicity of an insecticide and an herbicide in separate single species tests in the laboratory for *Daphnia* (Crustaceae) and *Scenedesmus* (green algae). Suppose there are no combination effects for each organism, because the pesticides used are acting highly specific. Thus, neither CA nor IA will predict any mixture toxicity for the crustacean or the algae. Now, what happens if you conduct the same experiment in an more realistic scenario, with the food web interaction also integrated in the experiment?

Of course, the algae serve as food for the crustacean. Thus, by depriving it of its food (herbicide action on the algae), a certain percentage of the daphnids may starve. Another percentage is killed by the insecticide. In sum, the effect of the treatment is higher than any model could predict from the single species tests. The importance of indirect effects in risk assessment was stressed in a review article by PRESTON 2002. Experimental evidence for the ability of detecting such effects in mesocosm studies is given by WENDT-RASCH 2003, PEITHER *et al.* 1996, or JAK 1996, for example.

As you can see, dealing with the impact of pesticides in a mixture is highly complex. Research on this topic is not too far advanced, so it is no surprise that it is not yet integrated in the regular risk assessment for new agents. Authorities in the US and Europe do not even agree over the use of mesocosm studies for the assessment of single substances. As noted above, in Europe such a study may be necessary, but this is not the case in the US. Mere laboratory work is regarded sufficient there (MAISE 2002). However, transferability of laboratory data to more realistic scenarios with single substances in risk assessment schemes has been discussed earlier

(e.g. PERSOONE and JANSSEN in HILL *et al.* 1994, HUBER and SCHINK 1994, SCHMIDPETER and HUBER 1990) and lead to the tiered approach proposed in EU 2002. Since interactions between organisms may even be more important with pesticide mixtures (see above), performing only laboratory studies is still more questionable when assessing the environmental risks of pesticide combinations.

1.4 Surveying literature data

For single species approaches, convincing results were found for the ability to predict mixture toxicity as well for the CA model as for the IA approach in algae and bacteria (ALTENBURGER *et al.* 2000, BACKHAUS *et al.* 2000a, FAUST *et al.* 2001). Little is known about the effects of combined pesticide treatment on multi-species systems (VIGHI *et al.* 2003). These were all laboratory studies. Data from field experiments for the prediction quality of these models is lacking completely.

To the knowledge of the author, only three studies have been conducted to examine mixture toxicity in outdoor mesocosm experiments. HINDELANG 1993 reported a more intense insecticide action (carbofuran) when applied together with a herbicide (atrazine). The DT_{50} was prolonged due to the decrease in the pH. FAIRCHILD *et al.* 1994 used atrazine together with esfenvalerate, a pyrethroid insecticide. They concluded that the combined treatment did not have different effects on zooplankton or fish than the insecticide treatment alone. However, they were not able to exclude that this finding was specific to their study. WENDT-RASCH *et al.* 2003 used the herbicide metsulfuron methyl together with cypermethrin (pyrethroid insecticide). They were able to characterize a series of secondary effects with both pesticides applied separately, but were unable to detect combination effects. The main reason for this was the domination of the zooplankton by Rotifers that were insensitive towards the insecticide at the concentrations used. Therefore they advised more studies on the topic with plankton communities that should be constituted of crustaceans.

In short, up to now combination effects have only been observable when a change in a functional parameter altered bioavailability of at least one pesticide. There are hints that secondary effects may cause major changes in the system reaction when (at least) two pesticides are applied jointly, but as yet there is no proof (see also the theoretical remark on daphnids and algae in 1.3). For this reason, a test in a multi-species systems that fully integrates the variety of biocoenotical interactions is needed to answer this question. Direct and secondary effects of each substance must be known in great detail to deduce combination effects. If there is any mixture toxicity, prediction of it should be attempted by using either CA or IA (depending on the pesticides used).

1.5 Approaches and objectives of this study

1.5.1 Choice of the test systems

Against above background, a series of mesocosm studies has been designed to clarify the question whether there are any combined treatment effects on plankton communities. From former studies (e.g. VOLM 1997, FUNK in prep.) it is known that the zooplankton in the ponds used is dominated by crustacean zooplankton. The effects on endpoints of such mesocosm

studies are fully comparable even between different years (GIDDINGS *et al.* 2001: consistent results in seven studies over a decade on two continents).

Mesocosm studies are intended to integrate a whole self-sustaining ecosystem. Food web interactions can therefore be examined in great detail. As mentioned above, little is known about variations of such interactions due to combined pesticide action because of the low number of studies conducted up to now. Emphasis was therefore put on secondary effects. The duration of the tests was two vegetation periods because such effects may well take a longer time to come into being and wear off again.

Endpoints of this study were populations of the plankton and several functional parameters like the pH or the conductivity. Additionally, macrophytes in the system were evaluated to some extend.

1.5.2 Choice of the pesticides

The pesticides used in this thesis are Isoproturon (IPU) and α-Cypermethrin (CYP). The first one is a phenylurea herbicide, the latter one a pyrethroid insecticide (PERKOW 1988). They were chosen for two major reasons:

1. Previous studies have been conducted at the same location with both of them (ESER 2001, HUBER *et al.* unpublished);
2. They have a totally independent mode of action: IPU is a photosynthesis blocker and CYP a neurotoxin (PERKOW 1988)

In this way, results are comparable with previous data *and* the pesticides perfectly meet the premises of the IA model. The first implication will provide strong arguments for the validity of the data derived by this study, the latter will allow giving a first estimate whether and to what extend a laboratory derived prediction model can be used for field data.

The big advantage of using so differently acting agents is that the effects can clearly be addressed to one of them, especially in the combination. A major focus of this work is set on secondary interaction via the food web, so it is essential to be able to tell which effect was caused by which agent. Using two pesticides that act more or less similarly would tend to blur interactions caused by each impact. The agents that were chosen here either have an impact rather at the top of the food web (CYP) or at its bottom (IPU). When investigating each substance alone, the reaction of the populations in the mesocosm to a stressor that predominantly alters interactions either from top-down or bottom-up can be derived. In the combination, it should therefore be possible to tell which impact is of greater importance and thus deducing a more general principle of the reaction to multiple stressors may be possible.

1.5.3 Questions to be answered by this work

The following questions are intended to be answered by this thesis:

1. What are the effects of CYP and IPU treatment on the plankton of an aquatic mesocosm?
2. In which way are these effects conveyed through the food web and through time?
3. What are the differences in these results when the two pesticides are applied jointly?
4. Can these differences be predicted and/or can a pattern for the system reaction be derived for combination effects?

After introducing the materials and methods used in this thesis the results of each part (CYP, IPU, and joint treatment) are presented and discussed. In the last part, answers to the questions above will be given.

2 Material and Methods

2.1 Artificial pond systems

2.1.1 General approach

In order to test the different effects of the two pesticides and their combination used in this thesis, two pond systems were set up:

1. The first one for the single substance studies in the year 2000; 14 enclosures were installed for the first two parts of this study focusing on the impacts of IPU (herbicide) and CYP (insecticide) applied separately. The pond system was additionally monitored in 2001.
2. The second mesocosm was installed for the combined application of IPU and CYP in 2001; in 2002 effects of this combination were recorded, too.

The approach can accordingly be divided in three parts: Insecticide part (CYP), herbicide part (IPU), and the combination of the two plant protection agents.

The pond systems are located at Grünschwaige Research Station, approximately 15 km from Freising, Bavaria. The surrounding area is used for agricultural research purposes by the Technische Universität München, Weihenstephan. Pond systems were generally arranged in accordance with HARAP 1999 and CLASSIC 2001 workshop guidance documents. Fish were not integrated in the test systems. Invertebrate predators are therefore on top of the food chain.

Figure 1: Mesocosm with enclosures for the single substance studies

These ponds are mesocosms according to HILL *et al.* (1994). They were installed in 1993 and had not previously been used for ecotoxicological studies; for details see VOLM (1997). In order to accomplish different concentrations of the pesticides used enclosures were introduced to the systems.

Table 1: Technical data of the artificial ecosystems

parameter	mesocosms	pond enclosure
material	0.8 mm stainless steel; inner layer 1 mm black polyethylene foil	stainless steel
volume	ca. 29,000 L	ca. 700 L
diameter	5 m	0.95 m
height	1.5 m	1.5 m
manufacturer	MTW Moderne Wassertechnik Gilching, Germany	Schorb Company, Moosburg, Germany

Macrophtyes were originally obtained from Hydrobaumschule U. Oldehoff (Achenmühle, Wolfratshausen) and from a lake in Scheyern (VOLM 1997). All plants integrated in the systems in this study were taken from other, uncontaminated ponds of the Fachgebiet Ökotoxikologie, TUM.

Both mesocosms used were re-arranged in autumn 1999. Excessive cover of *Potamogeton lucens* was removed and an approximately 5 cm thick sand layer put down on the sediment.

In April of the year each part of the study started (2000 and 2001, respectively), macrophytes were planted. For each enclosure five plastic containers with *Myriophyllum spicatum*, three with *Potamogeton natans* and *Elodea canadensis* were used, respectively. They were all planted in natural sediment of the Kirchdorfer Weiher (see DAWO 1993 for the biology of this small lake). More sediment and water of this pond (approx. 60 L all together) was integrated in the mesocosm to enrich the biocoenosis in each of the study parts.

In order to avoid cross-contamitation all sampling was done with increasing pesticide concentrations. Separate equipment was used for each of the study parts where it was possible.

2.1.2 Single substance studies

Macrophytes, natural sediment and water were introduced on April, 5^{th,} 2000. Enclosures were pressed in the sediment on June, 6th 2000, two weeks before the application of the pesticides. This procedure avoids leaking of pesticide residues (e.g. FUNK 1997) and gives the biocoenosis enough time to recover from the disturbance due to this treatment.

Since the macroinvertebrate community of the pond lacked Crustaceae, 10 individuals of *Asellus aquaticus* were introduced in each enclosure (see ROTH 2001) on the same day.

14 enclosures were used for this part of the study. Four of them served as uncontaminated controls, five for the CYP and five for the IPU study. Concentration levels were not duplicated; levels of active ingredient were 0.015 µg/L, 0.075 µg/L, 0.375 µg/L, 0.750 µg/L, and 1.875 µg/L CYP and 4 µg/L, 16 µg/L, 64 µg/L, 128 µg/L, and 256 µg/L IPU, respectively. The pesticides were added to the enclosures on June, 22nd 2000.

All sampling in treated parts started in the CYP enclosures. The equipment used in both the insecticide and the herbicide enclosures[1] was rinsed thoroughly between applying it to enclosures with the other active ingredient.

[1] electrodes for water quality parameters: Oxygen, conductivity, and pH

2.1.3 Combined application study

Macrophyte cover was reduced on October, 4th 2000 in order to ensure comparable growth conditions compared to the single substance parts. Planting of the macrophytes took place on April, 9th 2001 together with the integration of water and sediment of the Kirchdorfer Weiher.

A total of 14 enclosures was inserted on May, 22nd 2001, three weeks before the combined pesticide treatment. Four enclosures served as controls, the remaining ten received combined treatment (levels duplicated) of CYP and IPU

- 0.015 µg/L CYP + 4 µg/L IPU,
- 0.075 µg/L CYP + 16 µg/L IPU,
- 0.375 µg/L CYP + 64 µg/L IPU,
- 0.750 µg/L CYP + 128 µg/L IPU, and
- 1.875 µg/L CYP + 256 µg/L IPU.

The combined treatment was done on June, 14th 2001.

2.2 Physical and chemical water parameters

Water was taken with a sampler built by ZIERIS (1983), two water columns each time. They were mixed in a bucket to destroy possible stratification (separate buckets for controls and the different xenobiotika) and transferred to bottles. Water sampling and measurements started at a fixed point of time, 1.5 h after sunrise.

Both above procedures were used to exclude effects caused by diurnal rhythms: the vertical migration in plankton is light-controlled and therefore stratification phenomena can appear (SOMMER 1994); moreover, there is a distinct diurnal oscillation in e.g. oxgene content in littoral habitats (SCHWOERBEL 1999). Additionally, the amount of chlorophyll a in algae changes rather quickly and is light-depending (FOY 1987, GERHARDT, personal communication). So all in all it is more advisable to start at a fixed point of time after sunrise than at a fixed time of the day.

Temperature, oxygene content and saturation, pH, and conductivity were measured on site using WTW equipment. Alkalinity determination (CO_3^{2-}) was done according to SCHWOERBEL (1994) in the laboratory on the sampling day. Photosynthetic active chlorophyll a content was determined simultaneously with a delayed fluorescence kinetic photometer (e.g. KRAUSE and GERHARDT 1984).

Sampling took place weekly starting with day –9 a.t. (i.e. nine days before treatment; "a.t." means "after treatment") up to day 118 a.t. (one extra sampling on day 139 a.t.) in case of the single substance application and from day –15 a.t. to day 112 a.t. in the combined study, respectively. Additionally, measures were taken on day 3 a.t.. In the year following the application, sampling was conducted monthly from March to September. No chlorophyll a determination took place in that year of the studies. Some supplementary measurements were conducted in a weekly schedule before the introduction of the enclosures.

Total phosphorus (TP), soluble reactive phosphate (SRP), nitrate-nitrogen (NO_3-N), ammonium-nitrogen (NH_4^+-N), silicate, potassium (K^+), calcium (Ca^{2+}), sodium (Na^+), and total hardness were measured on day –9, -1, 14, 28, 55, 83, 111 and 139 a.t for the single

substance studies and on day –15, -1, 7, 14, 28, 55, 84, and 112 a.t. for the combined study. Methods are listed in Table 2.

Table 2: Methods used for water chemical parameters

Parameter	Method	limit of quantification (LOQ)
NH_4^+-N	Colorimetric as indophenol blue according to DIN 38 406 part 5 / DEV – 12. Supply 1983	0.01 mg/L
NO_3-N	Ion chromatography Dionex Serie 2000 i/sp Column: Dionex AS4A 4 mm (10-32) IonPac Guard column; Dionex AG4A 4 mm (10-32) IonPac Eluent: 0.6 mM Na_2CO_3, 0.57 mM $NaHCO_3$ Regeneration: 25 mM H_2SO_4 Flow rate: 1 ml/min	0.01 mg/L
SRP	Colorimetric according to DIN 38 405 (Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung)	0.001 mg/L
TP	Colorimetric according to DIN 38 405 (Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung)	0.001 mg/L
Total hardness	Titration with Titriplex (Merck)	0.5 °DH
Sodium, potassium, calcium	Flame photometer (Eppendorf, Fa. Netheler & Hinz, Hamburg)	1 mg/L, 0.1 mg/L, 1 mg/L
Silicate	Colorimetric, test set Aquamerck, Art. 8045 (Merck)	0.01 mg/L

All chemicals used were at least p.a. quality.

2.3 Biological sampling

Sampling methods did not change between the three parts of this thesis. Again, care was taken to avoid contamination (see above). Generally, sampling took place after the examination of water physics. It was constrained to a bi-weekly schedule (±1 day) after 14 days a.t. (single substances) and 28 days a.t. (combined approach) in the year of the application. Zooplankton of day –9 a.t. in the single substance studies could be not evaluated due to problems in sampling (too much sediment in the samples).

2.3.1 Zooplankton

Zooplankton communities were investigated via artificial substrates (Figure 2). They are well-established for macrozoobenthos (e.g. ROTH 2001, SANDMANN 2000, HUBER *et al.* 1995, BROCK and CRUM 1992) and work fine with Zooplankton as well (FUNK in prep., GRÜNWALD 2000, FUNK and HUBER 1999). Zooplankton organisms that are not clearly pelagic tend to orientate to submersed structures and to stay near them all the time (FLÖßNER 1972; VOIGT and KOSTE 1978; SCHWOERBEL 1999, BLINDOW and HARGEBY *et al.* 2000). A previous study (GRÜNWALD 2000) showed that in artificial pond systems with intense plant growth species preferring littoral-like habitats are predominant. Therefore, sampling of water surrounding a certain structure is preferable.

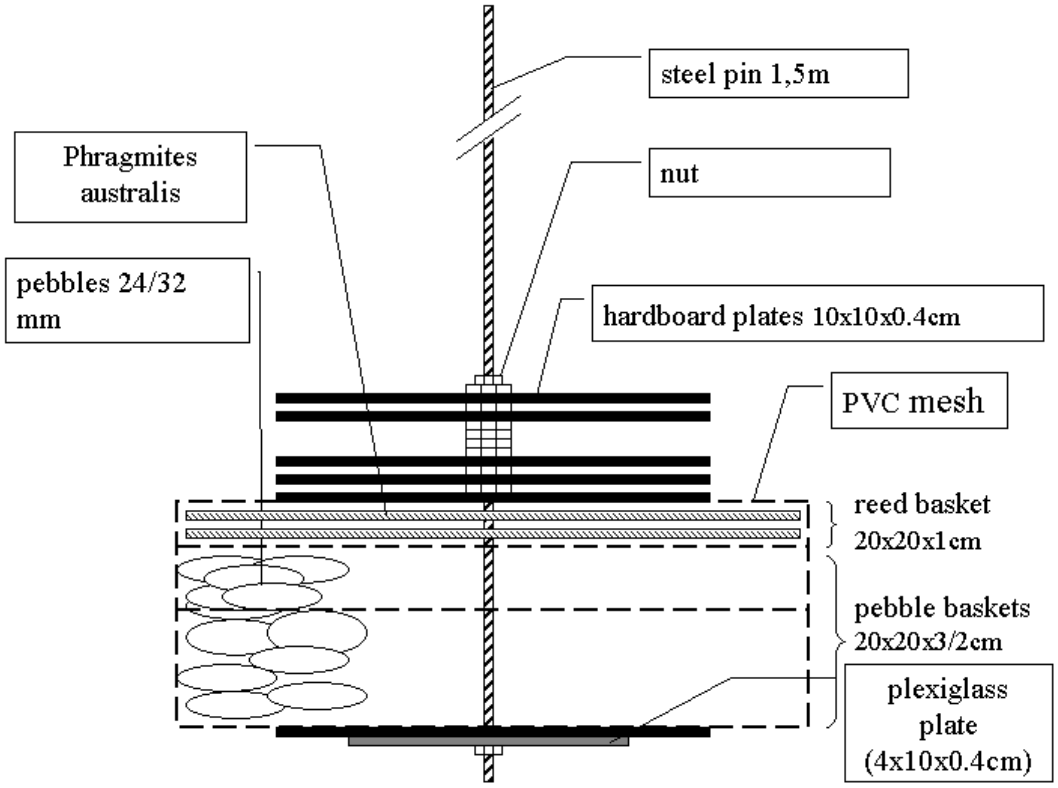


Figure 2: Construction of the artificial substrates

These substrates were set on ground pebbles baskets (PVC mesh, 20x20x2 cm) to avoid bigger amounts of sediment in the samples that hinder examination. Substrates were taken with a sampling device (ESER 2001, ROTH 2001, SANDMANN 2000) and caught organisms were rinsed onto a photo dish (separate ones for each concentration). Sampling started right after the water quality measures, i.e. at about 2 h after sunrise. Individuals of *Chaoborus crystallinus* were instantly counted alive. Sediment and detritus were allowed to sink to the ground of the dish. The water above the sediment was poured through a stainless steel sieve (63 µm). The remains of the sample were poured back to the corresponding enclosure. Animals in the sieve were transferred to PE containers and fixed with a 38% formaldehyde solution (resulting concentration app. 4%). They were counted in the lab using a stereoscopic microscope at 40x magnification.

These procedures ensure

- that there are only living organisms (at the time of sampling) in the fixed sample: Those not capable of swimming are excluded;
- avoiding deviations caused by vertical migration;
- catching a good sample of the organisms which are most important in the simulated ecosystem.

2.3.2 Phytoplankton

Phytoplankton was taken from the water samples for water quality parameters (2.2 page 24). About 200 ml were fixed with app. 20 ml Lugol's solution. For microscopic analysis an aliquot was taken and the algae in it were allowed to settle in a sedimentation chamber (Utermöhl, see

SCHWOERBEL 1994). One row of the chamber was examined; organisms were determined to the lowest possible taxonomic level.

2.3.3 Biomonitoring

Biomonitoring was performed with selected species to get an idea when the pesticide residues have become small enough to permit recovery or colonization.

Water was taken with the integrated sampler (see 2.2 for details) from each enclosure and poured through a sieve (63µm). An aliquot of app. 1 L was poured into glass beakers, one for each enclosure. Everything that was kept back by the sieve was poured back into the respective enclosure. For CYP and the combined study no sieving was done to avoid adsorption of the active ingredient to the sieve and thereby reducing its concentration.

Information on animals used for the single substance studies:

Chaoborus crystallinus was taken from an uncontaminated pond on the test site by net sampling. Individuals were put to photo dishes to let the animals adapt to the new environment. Only animals in good condition were taken for the assessment.

Daphnia pulex and *Simocephalus vetulus* were derived from laboratory cultures: Animals from split pond systems were kept at 16 h of light, 18°C for approx. one month. They were cultured in 63 µm sieved pond water and fed on *Scenedesmus subspicatus*, *Monoraphidium contortum* and *Chlamydomonas sp.*

In the combined study all animals were taken from an uncontaminated pond on the test site: *Simocephalus vetulus*, *Eudiaptomus gracilis*, and *Chaoborus crystallinus*. They again were transferred to photo dishes and only healthy ones chosen for the tests.

Each beaker was equipped with 10 animals of choice:

Cladocerans: Adults with eggs,

Adult Copepods without egg packages,

and *Ch. crystallinus* of size 0.5-1 cm.

The animals were transferred to very small glass dishes and poured into the test beakers. Since the additional amount of uncontaminated water is very small (approx. <1%), no relevant concentration deviation is expected. Monitoring followed the scheme in Table 3:

Table 3: Time schedule biomonitoring

time a.t.	organisms used	
	single application	**combined study**
6h	*S. vetulus, Ch. crystallinus, D. pulex*	*S. vetulus*, *E. gracilis*
7d	*S. vetulus, Ch. crystallinus, D. pulex*	*S. vetulus, Ch. crystallinus,*
20d		*Ch. crystallinus*
28d		*Ch. crystallinus*
35d		*Ch. crystallinus*
41d		*Ch. crystallinus*

All beakers were evaluated for lethal effects 24 h after integration of the animals. Additionally, the 6 h a.t. experiments were looked at 70 h after their starting time.

2.3.4 Laboratory studies

Single species tests were performed with 10 individuals of *S. vetulus* and *Eu. gracilis* for each test beaker. They were taken from uncontaminated ponds and kept at least two week in the climate chamber (see for 2.3.3 details). The matrix for all experiments was 1 L of 63 µm sieved, uncontaminated pond water.

Screening for Isoproturon effects was done with concentrations of 0, 500, and 1000 µg/L active ingredients (a.i.). A commercially available herbicide (Stefes IPU 500) was used. Effects were recorded 24 h and 48 h a.t.

CYP effects were monitored at 0, 0.2, 0.5, 1, and 2 µg/L, having each concentration duplicated. Dead individuals were recorded 24 h and 48 h a.t..

2.3.5 Macrophyte mapping

On the test site an orthogonal reproduction of the macrophyte covered area was recorded of each enclosure on the day of the application and as well as on day 90 a.t. and 364 a.t. (single substances) and day 82 and 362 a.t. (combined application), respectively. These diagrams were digitalized and the total covered area measured via the program "Image-J 1.29x" (W. RASBAND, National Institute of Health, USA). Values are expressed as percentage of the whole enclosure area.

2.4 Pesticides

The pesticides used were chosen in order to have a totally different mode of action. As an advantage, other studies have already been conducted with them in comparable mesocosms of the Fachgebiet Ökotoxikologie (ESER 2001, HUBER *et al.* unpublished, DAWO in prep.)

For correct dosage, water volume of each enclosure was calculated using the mean of two measurements of the depth and the diameter.

Application took place on June, 22nd 2000 for the single substance study and on June, 14th 2001 for the combined application.

Concentration levels (active ingredient, a.i.) in the single application were 0.015 µg/L, 0.075 µg/L, 0.375 µg/L, 0.750 µg/L, and 1.875 µg/L CYP and 4 µg/L, 16 µg/L, 64 µg/L, 128 µg/L, and 256 µg/L IPU, respectively. They are also referred to as "CYP1"-"CYP5" and "IPU1"-"IPU5".

The combined treatment enclosures received levels of 0.015 µg/L + 4 µg/L, 0.075 µg/L + 16 µg/L, 0.375 µg/L + 64 µg/L, 0.750 µg/L + 128 µg/L, and 1.875 µg/L + 256 µg/L CYP + IPU (Level/Lv/Step/S 1-5).

All solutions were prepared with bi-distilled water and thorough ultrasonic treatment.

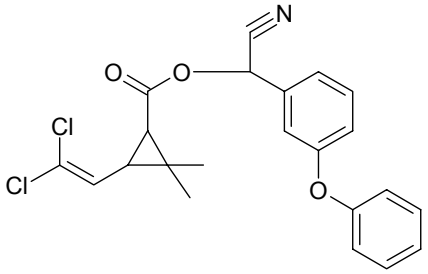
2.4.1 Alpha-Cypermethrin (CYP)

CYP used as active ingredient in the presented studies stemmed from a commercially available product, FASTAC SC ®, BASF Corp. (formerly American Cyanamid). It is a synthetic pyrethroid insecticide. The active ingredient is also know as "Alphamethrin".

2.4.1a Technical data

Technical data provided by the manufacturer and found in PERKOW (1988), TOMLIN (1997) is listed in Table 4

Table 4: technical data of CYP

Data	
Product description	pyrethroid insecticide
Test substance:	FASTAC® SC insecticide
Active ingredient (a.i.):	alphacypermethrin, AC 900049
Structure:	
Source:	Cyanamid Agriculture Limited, Gosport, UK
CAS Number:	67375-30-8
Formula Ref:	CF 06677
Batch No:	166772
Content of a.i.:	101.7g/L
n-octanol/water log P_o/w	6.94 (pH 7)
Color:	white
Physical State:	viscous homogeneous liquid
Density:	1.0305 g/mL
Solubility in Water:	formulation mixes with water (forms a suspension)
Storage Conditions:	dry, at room temperature
Application against	a wide range of chewing and sucking insects in fruits, lice in hop, etc.
Mode of action:	non-systemic insecticide with contact and stomach action. It acts on the central and peripheral nervous system of the target organisms in very low doses
Toxicity	LD_{50} (oral) in rats 70-400 mg/kg, LD_{50} (24 h) in bees 0.059g/bee, LC_{50} (96 h) in rainbow trout 2.8 µg/L. LC_{50} (48 h) *Daphnia*: 0.1-0.3 µg/L non-phytotoxic

2.4.1b Preparation and application of the insecticide solutions

A stock solution was prepared with the amount of the active ingredient for the highest treatment level (1.875 µg/L) contained in 50 ml (i.e. it is simultaneously the application solution for this level). Therefore, the mean volume of all enclosures was computed. All other application solutions were diluted from this stock solution. Concentration, again, were chosen to have the amount of CYP in a 50 ml aliquot. For the nominal concentration in the enclosures please see 2.4 (above). Application was performed with 50 ml pipettes; separate ones for each treatment level. The solutions were directly added to the water surface as recommended in HUBER *et al.* (1995) and CLASSIC (2001).

2.4.1c Analysis of pesticide residues

This analysis was performed by the "Landwirtschaftliche Hauptversuchsanstalt (HVA)", Freising, Weihenstephan, using GC-ECD detection. Samples were taken from the three highest treatment levels 6 h, 3 days, 7 days and 14 days a.t. Water was taken with the sampler like the one used by ZIERIS (1983) and immediately transferred to glass bottles; 1 L each. Transport to the HVA took place in dark insulated bags with cooling elements on the sampling day (single substance study). In the combined study, samples were frozen. Since some of the sample bottles broke during this process they had to be defrosted and transferred to intact equipment for transport. Analysis was therefore restricted to the 6 h a.t and the 3 days a.t. samples.

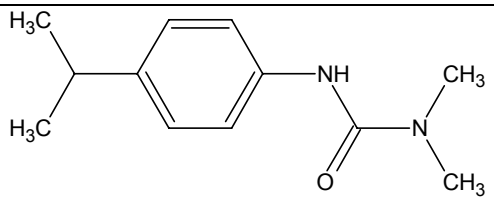
2.4.2 Isoproturon (IPU)

No formulation but the "technical" product was used in the mesocosm studies. In the single substance studies it was provided by Riedel-De-Haën, in the combined study by Ehrenstorfer. Both products are of comparable quality (purity >99.5%). A review on the mode of the action and degradation can be found in ESER (2001).

2.4.2a Technical data

Data of Table 5 found in PERKOW (1988), TOMLIN (1997), ARNAUD, TAILLANDIER *et al.* (1994).

Table 5: Technical data of Isoproturon

Data	
Active ingredient	N-(4-Isopropylphenyl)-N',N'-dimethylurea
Sum formula	$C_{12}H_{18}N_2O$
Molar weight	206.29 g/mol
Structure	
Solubility at 25 °C in water	170 mg/l
n-octanol/water log P_o/w	ca. 2.5 (pH 7, 22°C)
melting point	151-153°C
Toxicity	LD_{50} (oral) female rat 2417 mg/kg, not harmful to bees, LC_{50} (96 h) *Lesbites reticulatus*: 90 mg/L, *Carassius auratus* 100 mg/L LC_{50} (48 h) *Daphnia*: 507 mg/L, LC_{50} (72 h) Algae: 0.03 mg/L
Mode of action	Inhibition of the photosystem II (D1 protein)

2.4.2b Preparation and application of the herbicide solutions

For each enclosure a separate flask with the application solution was prepared. Amounts for the three lower treatments (IPU 1-3) were diluted in 1 L bi-distilled water. The forth treatment level solution (i.e. for IPU 4) was produced in 2 L and the highest (IPU 5) in 5 L flasks,

respectively. These amounts of water were necessary not to exceed the maximum solubility of IPU.

The preparations were stirred for three days in the dark and put into an ultrasonic bath every now and then to ensure complete solution of the herbicide (see also ESER 2001).

Contents of the flasks, again, were added directly to the water surface. Flasks were rinsed with enclosure water to ensure complete transfer.

2.4.2c Analysis of pesticide residues

Samples were taken 6 h a.t., 3, 7, 14, 30, 92, and 278 days a.t. (single substances) and 6 h, 3, 7, 14, 28, 55, 84, 112, and 362 days a.t. (combined study). Sampling method see 2.4.1c.

Aliquots of 200 ml (IPU1-3) and 100 ml (IPU4-5) were enriched on C18-cartridges (Sep Pak plus, Waters) using a vacuum system (Supelco) on the sampling day. The cartridges were conditioned with 5 ml acetone (HPLC grade), analytes eluted with 4 ml acetone. The solvent was then allowed to evaporate under a stream of nitrogen in a water bath (40°C). Samples were kept refrigerated until residue analysis.

Residues were dissolved in bi-distilled water (1-10 ml). Analysis followed SCHUELEIN *et al.* (1996) and ESER (2001); both methods modified.

All samples but the one of day 278 of the single substance study were measured once on a HPLC device, HP Series 1500, with quartanery pump, automatic degaser and UV detection device. Details see Table 6.

Table 6: Detection parameters IPU measures in 2000

Parameter	value
Analytical column	Nucleosil 5, C18, Et 250/8/4, Macherey und Nagel 720014, with a guard column
UV detection	λ=240 nm
Eluent	acetonitril : water 70:30 (v:v) isocratic; gradient grade
flow rate	0.8 ml/min
Calibration	external standards
Detection limit	approx. 12 µg/L without preconcentration step
Retrieval of IPU residues	>95% with SPE step
Sample injection volume	50 µL

All other samples were measured twice on a Kontron HPLC system: LC pump 410, UVIKON 720 LC UV/VIS detector with a slave computer. Details see Table 7. Mean values were taken into interpretation.

Table 7: Detection parameters IPU measures in 2001

Parameter	value
Analytical column	Supelco Supelcosil LC 8, 15 cm x 4.6 mm; 5 µm
UV detection	λ=240 nm
Eluent	acetonitril : water 40:60 (v:v) isocratic; gradient grade
flow rate	0.8 ml/min
Calibration	external standards
Detection limit	<25 µg/L without preconcentration step
Retrieval of IPU residues	>96% with SPE step
Sample injection volume	20 µL

2.5 Statistical evaluation of data

2.5.1 CYP residue analysis

DT_{50} values were calculated for the single substance study using the curve fitting tools of Microcal ORIGIN 6.0, regression model: $y = m * \ln(x) + y0$ with *m:* slope of the curve, *y0*: initial concentration (time=*x*=0). DT_{50} values of the same data were also calculated by ROTH (2001) using a different regression model. In the combined study, due to the discrepancies in sample storage (see 2.4.1c), no DT_{50} values were determined. The data were assessed with regard to the initial concentrations.

Extrapolations of a.i. concentrations for points of time integrated in CCA analysis (see 2.5.8b) with no direct measurement were performed using the model of ROTH 2001 for the single substance study. Data of the combined treatment were estimated using the results of the biomonitoring study (see 2.5.3).

2.5.2 IPU residue analysis

DT_{50} values were calculated using the curve fitting tools of Microcal ORIGIN 6.0. Regression model was $y = m * \ln(x) + y0$ (see also 2.5.1).

Extrapolations on concentrations using the equation above were done for the CCA analysis (2.5.8b below). In this analysis it is very important to know at least estimated values for the concentration of the active ingredient on all sampling dates that enter the statistics. Therefore, values *y0* and *m* for each treatment level were used to compute concentration data when it was not assessed directly via HPLC.

2.5.3 Biomonitoring

Counts of dead animals were plotted against the initial nominal a.i. concentrations (logarithmic scaling) for all tests. LC_{50} values were obtained using a sigmoid fit, dose-response model with minimized chi-square option switched on (Microcal Origin 6.0). Additionally, log-linear fits were also performed (regression model $y = m * \log(x) + y0$ with *x*=[a.i.], *y*=counts). In these cases, only the log-linear part of the data points was integrated into analysis.

Dissipation of CYP in the combined study was estimated using the LC_{50} data from above. The reason for this is the need of concentration data in the CCA (2.5.8b). No direct

measurements of CYP were performed here (see 2.5.1). Estimations were performed with the monitoring data of *Chaoborus crystallinus* in the following way:

The LC_{50} computed with the initial CYP amount on one specific point of time ($LC_{50}^{time, real}$) is divided by the LC_{50} from laboratory single species tests (with exact concentrations used for this), $LC_{50}^{nominal}$. Thereby a factor is produced for each date when biomonitoring took place. The $LC_{50}^{nominal}$ was set to 0.015 µg/L (Data from FUNK and HUBER, personal communication). Afterwards, dividing the initial nominal concentration by this factor, an estimation for the real concentration in the enclosure water at the biomonitoring date is obtained.

These concentration estimates were plotted against time. Regression analysis was performed using an exponential model (Microsoft EXCEL): $y=y0*e^{(-a*x)}$, with x=time, y=concentration, y0= initial concentration (time=0), and a=constant. This was done for each initial concentration level. With these curves giving a proper fit, concentration estimates for arbitrary points of time can be calculated. A DT_{50} value for these estimates was determined as well. Because of the algorithm used this must be a common one for all concentration levels.

The quality of the resulting concentration estimates can be assessed by comparing the constant *a* of each a.i. level to the initial nominal amounts of CYP.

These calculations were appropriate because *Ch. crystallinus* is not (directly) affected by IPU (see results of the IPU biomonitoring in the second part of this thesis). All toxic effects are therefore linked to CYP action.

2.5.4 Single species tests

LC_{50} data were calculated using the sigmoid dose-response model of Microcal ORIGIN 6.0.

2.5.5 Plankton abundance data

Abundance of planktonic taxa were plotted against time. Temporal maxima of some species can't be surveyed easily in a common line plot. Therefore, additional surface plots were used in these cases.

The most dominant species were determined (KLOFT AND GRUSCHWITZ 1988):

$$\text{Dominance}\left(taxon\,1\right)=\frac{\text{Number of individuals } taxon\,1}{\text{Number of taxa in sample}}*100\%$$

The widely used index figures Shannon's index and evenness were calculated using the formulae given in LUDWIG & REYNOLDS 1988. Additionally, the RAD index was used to compare similarity between treatment groups (WHITTAKER 1952 in LUDWIG & REYNOLDS 1988, LEGENDRE & LEGENDRE 1998). This index has the "distance" between the compared groups (control mean and treatment level) in focus. Identical samples score "0", and totally different ones "2".

Treatment effects were classified according to the system proposed by BROCK *et al.* 2000 in EU 2002:

Class 1: "effect could not be demonstrated"

- No (statistically significant) effects were observed as result of the treatment, and
- observed differences between treatment and controls show no clear causal relationship.

Class 2: "slight effect"

- Effects reported in terms of "slight" or "transient" and/or other similar descriptions, and
- short-term and/or quantitatively restricted response of sensitive endpoints, and
- effects only observed at individual samplings.

Class 3: "pronounced short-term effect"

- Clear response of sensitive endpoints, but total recovery within 8 weeks after the last application, and
- effects reported as "temporary effects on several sensitive species", "temporary elimination of sensitive species", "temporary effects on less sensitive species/endpoints" and/or other similar descriptions, and
- effects observed at same subsequent sampling instances.

Class 4: "pronounced effect in short-term study"

- Clear effects (such as strong reductions in densities of sensitive species) observed, but the study is too short to demonstrate complete recovery within 8 weeks after the (last) application.

Class 5: "pronounced long-term effect"

- Clear response of sensitive endpoints and recovery time of sensitive endpoints is longer than 8 weeks after the last application, and effects reported as "long-term effects on many sensitive species/endpoints", "elimination of sensitive species", "effects on less sensitive species/endpoints" and/or other similar descriptions, and
- effects observed at various subsequent sampling days.

2.5.6 NEC Analysis

Parameters were plotted against time. For each sampling date after the application of the test substance(s) a linear regression was performed using the linear part of the plot: Log (a.i.) against sample value. From the resulting regression equations a NEC value (No Effect Concentration, see LIBER *et al.* 1992, ESER 2001) was calculated whenever the coefficient of determination (R^2) was higher than 80% (biological data) or 90% (water quality data). The NEC is the crossing point of the regression curve with the control mean, and the 95% confidence intervals of the control data (upper and lower limits of the NEC). Since in PRC analysis (see 2.5.8a) all controls are set to zero by its algorithm, canonical coefficients (cdt values) before the application were used as control data.

Mean values of the NEC were computed for the year of the application.

2.5.7 Calculations of NOECs

NOEC values (No Observed Effect Concentrations) were gained by William's tests (WILLIAMS 1972). Abundance data were (log+1) scaled before analysis. All other data were entered directly into the program TOXSTAT 3.0.

A $NOEC_{community}$ can be derived from multivariate analysis (see 2.5.8 for details on these methods). PRC analysis provides information about whether there is any significant effect of

the treatment regime, but cdt values can't be tested directly for a NOEC because of the lack of control data (among other reasons, see VAN DEN BRINK & TER BRAAK 1999). This can be circumvented by applying PCA analysis (Principal Component Analysis, LEGENDRE & LEGENDRE 1998) on the "species data" for every sampling date if the PRC is "significant" for this data set. Sample scores of the first canonical axis are then tests by William's test for significant deviation (VAN DEN BRINK & TER BRAAK 1999).

The highest a.i. level giving no significant deviation ($p<0.05$) from the controls is defined NOEC. Values are only considered valid if they were identical on at least two consecutive sampling dates.

Please note the use of the terms "NOEC" and "N(O)EL" (no (observed) effect level) in this work. NOECs are always those values determined by applying the Williams' test procedure on them. The other term is used when a concentration was chosen either by combining several analyses' NOECs/NECs or a "real" NOEC was not calculated. The level where no effect occurred was then derived by NEC analysis alone or by thorough investigation of the development of the parameter in question.

2.5.8 Multivariate statistics

2.5.8a Principal response curves (PRC)

These curves have been computed with CANOCO for Windows 4.0 (VAN DEN BRINK & TER BRAAK 1998, 1999). A fairly good synopsis is given in ESER 2001. Quality of the statistics is ensured by Monte Carlo permutation test (preventing random results, $p<0.05$). The results of this analysis, the "cdt values", are plotted against time for each treatment level. All controls score zero (i.e. they are represented by the x-axis). Reaction of certain taxa to the treatment can be derived by looking at "species score". Negative scores indicate that abundances react inversely to the progression of the cdt values, i.e. if the curve is rising, the abundance of this taxon is decreasing. Taxa are regarded important for the community reaction towards the treatment if their species score is higher than 0.5 (absolute value).

2.5.8b Canonical Correspondence Analysis (CCA)

Again, this type of exploration was done with CANOCO for Windows 4.0. The big advantage of this method is its combined examination of abundance and environmental data. Changes in community structure can be linked to abiotic data (so called "factors"). The importance of a certain factor for a specific sample or a species can be easily derived from joint plots (TER BRAAK 1987, TER BRAAK and VERDONSCHOT 1995, LEGENDRE and LEGENDRE 1998)

2.5.9 BLISS independence and Index of Prediction Quality (IPQ)

BLISS independence is a model for interaction of substances with dissimilar mode of action (BLISS 1939, BERENBAUM 1985, GRECO *et al.* 1995, ALTENBURGER *et al.* 1996, WALTER 2002). In the presented thesis, this requirement is surely met using a neurotoxin and a photosynthesis inhibitor (2.4).

For prediction of abundance changes under treatment regime, per cent values of dead individuals at the experimental a.i. concentrations (C_x values) were calculated for both application types (single/combined) for every sampling date and treatment level:

$$C_X = 1 - \left(\frac{treated}{control} \right)$$

The C_X values of the single substances were used to calculate a combined effect:

$$C_{X,combined} = 1 - \left[\left(1 - C_{X,CYP}\right) * \left(1 - C_{X,IPU}\right) \right]$$

This equation was adapted from WALTER 2002.

Trends in water quality parameters were simulated in a slightly different way. Simple deviations from the control mean at the given a.i. amounts (D_x) were calculated:

$$D_X = \frac{treated}{control}$$

These D_x values where then entered in the BLISS model:

$$D_{X,combined} = 1 - \left[\left(1 - D_{X,CYP}\right) * \left(1 - D_{X,IPU}\right) \right]$$

Calculations were only applied when a trend in C_x/D_x data of at least one of the single applications was visible, showing either an increase or a decrease. Up to this point, calculations are somewhat similar to the "Fractional Product Method" of WEBB 1963 (in GRECO *et al.* 1995). Instead of claiming synergism or antagonism in the combined treatment, the model "BLISS independence" is evaluated for its ability to predict the mixture effects.

Hence, assessing the prediction quality was done by applying the IPQ (Index of Prediction Quality, ALTENBURGER *et al.* 1996, GRIMME *et al.* 1994) on calculated and observed combination toxicity:

$$C_{predicted} > C_{observed} : \; IPQ = \frac{C_{predicted}}{C_{observed}} - 1$$

$$C_{predicted} < C_{observed} : \; IPQ = -\left(\frac{C_{observed}}{C_{predicted}} \right) + 1$$

Instead of using the C_x values, the D_x were used in the same equation for the water qualtity parameters.

Thus, IPQ > 0 means the model underestimates the combined effect. IPQ=0 suggests an exact hit of the combined impact, and IPQ < 0 means an overestimation of the effect.

3 Results and discussion of the α-Cypermethrin study

3.1 Insecticide residues

Data for the amounts of active ingredient in the outdoor enclosures are listed in Table 8.

Table 8: CYP residues in the single substance study

time [days]	0, nominal	0.4	3	7	14	DT_{50} [days]
CYP3 [µg/l]	0.375	0.2	0.04	below detection limit		1.99
CYP4 [µg/l]	0.75	0.57	0.07	below detection limit		1.88
CYP5 [µg/l]	1.875	1.06	0.38	0.06	0.008	3.23

mean value (DT_{50})= 2.36±0.75 days

Nominal concentrations (time=0) were not included in the regression analysis. Statistical data (quality of the regressions) is presented in Table 9.

Table 9: Regression analysis of CYP residues, model $y = m * \ln(x) + y0$

level	m	y0	R	SD	p
CYP3	-0.146	0.2	-1	0	<0.0001
CYP4	-0.456	0.57	-1	0	<0.0001
CYP5	-0.409	0.958	-0.96	0.16	0.039

On the first sampling, a mean of 62% of the toxin was recovered. These findings are much better than in GRÜNWALD 2000 and SANDMANN 2000, who found about 20%-30% of the initial amount after six hours. Together with the fast dissipation from the water column it is concluded that the intended treatment levels were met. The DT_{50}-value is perfectly in line with DUTTON and PEARSON 1987. They stated a duration of about 2.8 days. Dissipation rates in SANDMANN 2000, GRÜNWALD 2000 and HUBER *et al.* (unpublished) were faster; these studies found a DT_{50} of about 15-20 hours. ROTH 2001 calculated roughly 1.55 days with the data presented here using a different regression model. The differences may be explained by the regression model used.

The active ingredient even in CYP5 (1.875 µg/L a.i.) is completely lost from the water column within about three weeks. The products of decomposition are less toxic (some orders of magnitude!) than the a.i. (HILL 1985). Direct impact on organisms is consequently limited to about two weeks after the application; there is still about 0.008 µg/L CYP in the highest level after fourteen days.

3.2 Single species tests

Single species tests were conducted with two different crustaceans, one Copepod (*Eudiaptomus gracilis*), and one Cladoceran (*Simocephalus vetulus*). Together with the Ostracods, these are the most important orders of crustacean zooplankton in the test system (see 3.7). Table 10 summarizes the results with the Copepod.

Table 10: Results of the single species test with *Eu. gracilis* on FASTAC SC

Eudiaptomus gracilis	24 h		48 h	
parameter	value	error	value	error
Chi^2	3.30		1.92	
start (A1)	9.82	1.21	9.93	0.93
end (A2)	3.60	5.05	0.33	3.37
LC50 [µg/L a.i.] (x0)	0.82	0.92	0.75	0.38
order (p)	1.83	2.61	1.82	1.21

The analysis on 24 h data does not give convincing results for a LC_{50} (see the error value). Calculations for 48 h are much better, LC_{50} being approx. 0.75 µg/L a.i.. The value for 24 h becomes more realistic by this outcome as well because it is fairly near the 48 h LC_{50}. Indeed, one would expect the LC_{50} (24h) to be higher because exposure time is shorter. A plot with the 48 h data is shown in Figure 3.

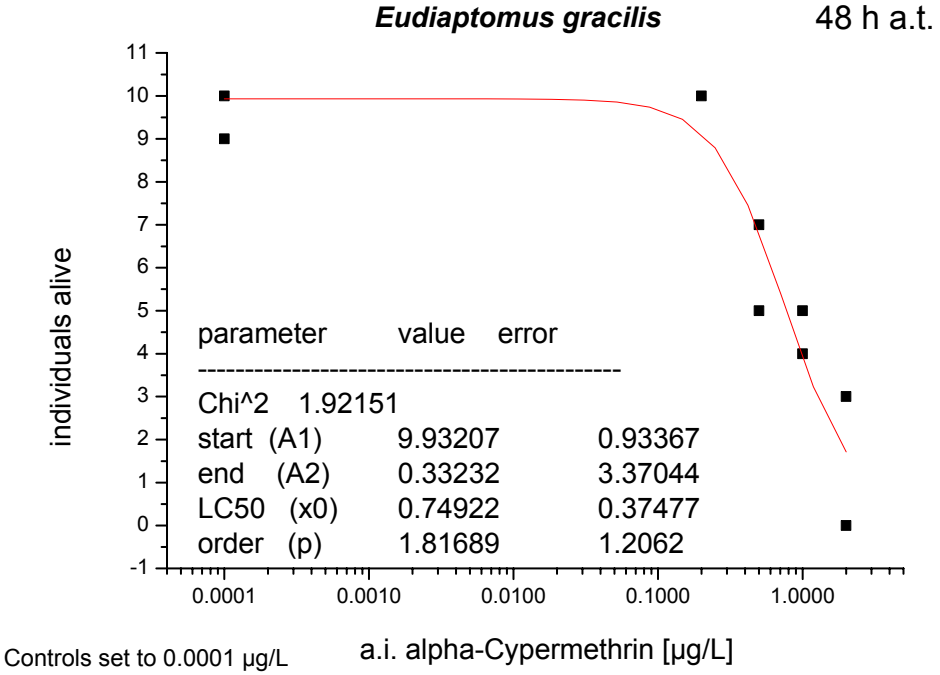


Figure 3: *Eudiaptomus gracilis* in the single species test with CYP, 48 h data

The Cladoceran showed a similar reaction to CYP, see Table 11.

Table 11: Results of the single species test with *S. vetulus* on FASTAC SC

S. vetulus	24 h		48 h	
parameter	value	error	value	error
Chi^2	0.63		5.02	
start (A1)	9.50	0.40	8.84	3.20
end (A2)	2.75	0.40	99593.11	$1.90*10^{10}$
LC50 [µg/L] (x0)	0.52	445371.30	$5.45*10^{11}$	$2.77*10^{17}$
order (p)	41.71	$1.20*10^{9}$	0.38	2.91

Again, these data have to be dealt with care. Only the 24 h values can be taken into further consideration. Note the chi square of the 48 h analysis: The data is not far from being significantly different from the model used from calculating the LC_{50} (df=5, 5% value is thus 11.07). Error values are also contributing to this interpretation.

Compared to *Eu. gracilis*, *S. vetulus* is more sensitive to the active ingredient: LC_{50} (*S. vetulus*, 24 h) ≈0.500 µg/L < : LC_{50} (*Eu. gracilis*, 24 h) ≈ 0.750 µg/L.

3.3 Biomonitoring

Compiled LC_{50} data for the three species can be found in Table 12.

Table 12: LC_{50} values [ng/L] for some species derived from biomonitoring data

Taxon	6 h a.t, 24 h	6 h a.t, 70 h	7 d a.t, 24 h
Ch. crystallinus	(13.16±2.37*10^8)	n.n.	13.74
S. vetulus	139.10±23.59	17.10±3.46	no effect
D. pulex	n.n.	717.25±3.94	no effect

Details of the experiments are summarized below.

3.3.1 Chaoborus crystallinus

In Table 13, data of the experiment with water taken 6 hours a.t. (24 h evaluation) are presented. Sigmoid regression analysis was performed on the data.

Table 13: Statistical data of the 6 h a.t (24 h evaluation) biomonitoring experiment the *Ch. crystallinus* (CYP study)

parameter	value	error
Chi^2	11.75	
start (A1)	6.75	1.71
end (A2)	0	1.76
LC50 [µg/L] (x0)	0.01316	0.00237*10^8
order (p)	13.39	1.84*10^9

This analysis can only be interpreted as "hinting" a toxic effect, because

- the number of dead animals in the control was too high (app. 34%, up to 10% would be acceptable), and
- the effecting LC_{50} (0.013 µg/L) is lower than the lowest concentration in the test (together with the bad chi^2-value, significantly different on the 5% level)

Because of the problem with the controls, no evaluation was done after 70 h.

The test starting seven days a.t. led to better results (Figure 4):

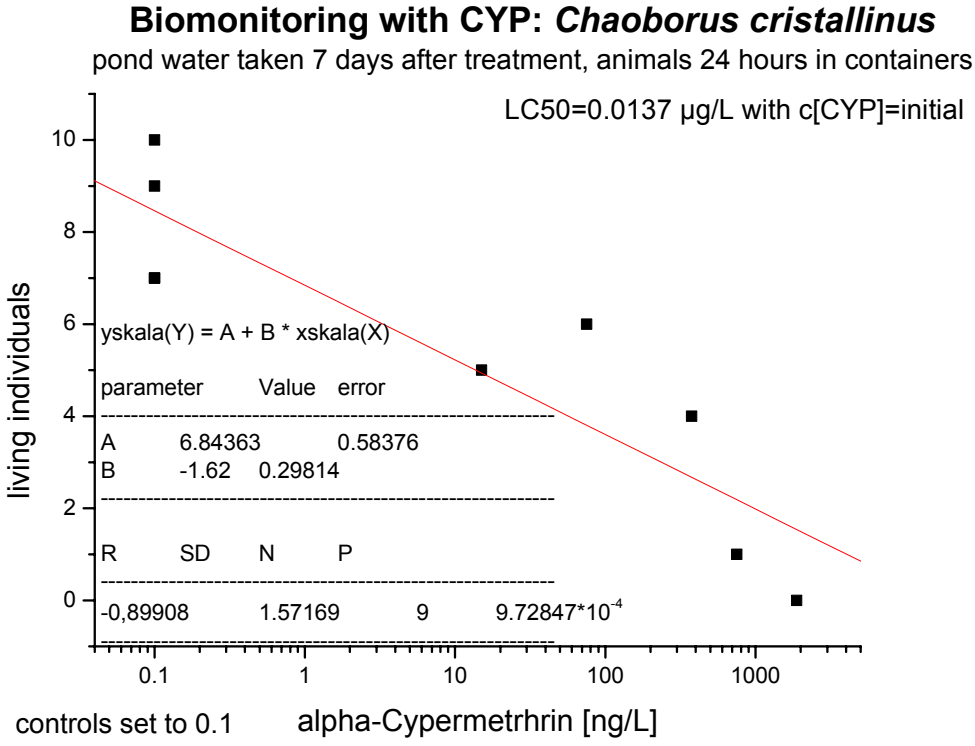


Figure 4: Biomonitoring with *Chaoborus crystallinus*: 7 d a.t., 24 h evaluation

Using a log-linear regression model, LC_{50} is approx. 0.014 µg/L CYP. Hence, the dipteran larvae is a very sensitive species to CYP.

These LC_{50} are corroborated by a laboratory value (24 h) of 0.015 µg/L (FUNK and HUBER, personal communication).

The amount of CYP still present in the water is 0.008 µg/L in CYP5 after 14 days. Re-colonization should be possible for *Chaoborus* from this point of time on and even earlier in the lower treatment levels. Minor toxic effects cannot be excluded, though.

3.3.2 Simocephalus vetulus

Here, comparisons to the single species laboratory data (see 3.2) can be made. Table 14 shows the results of the 6 h a.t. experiments.

Table 14: Results of the biomonitoring test with *S. vetulus* on FASTAC SC (6 h a.t. experiment)

S. vetulus	24 h		70 h	
parameter	value	error	value	error
Chi^2	0.22		0.18	
start (A1)	9.83	0.21	9.86	0.26
end (A2)	0.28	0.42	0.35	0.45
LC50 [µg/L] (x0)	0.14	0.02	0.02	0.003
order (p)	2.24	0.51	0.88	0.23

The seven days a.t. test showed no toxic effect at all for *S. vetulus*. No toxic effects are limiting its abundance from that time on (at least the life stage tested here).

The LC_{50} found in this test (≈ 0.14 µg/L) is more than three times lower than the laboratory one (≈ 0.52 µg/L, see Table 11).

Figure 5 is a graphical representation of the data.

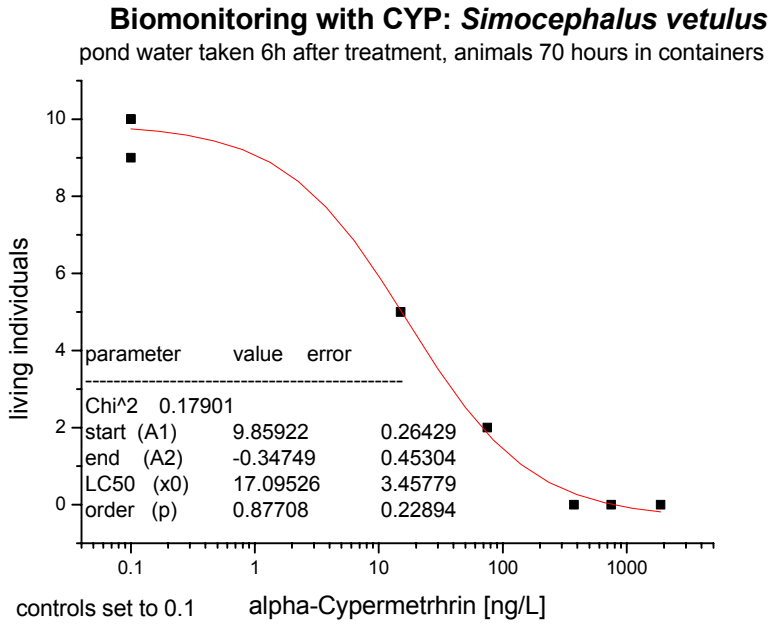


Figure 5: Biomonitoring with *S. vetulus*, 6 h a.t., 70 h examination

3.3.3 Daphnia pulex

Evaluation of the experiments with *D. pulex* is given in Figure 6.

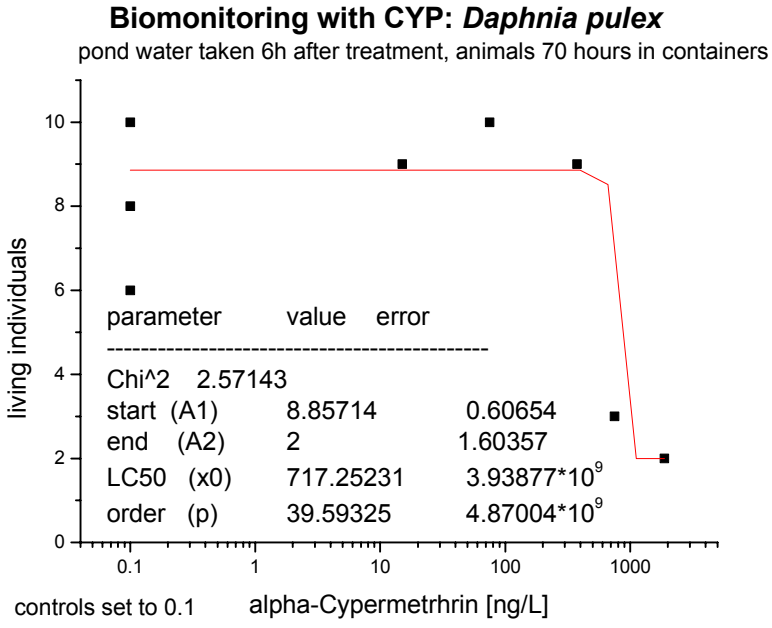


Figure 6: Biomonitoring with *D. pulex*, 6 h a.t., 70 h examination

The LC_{50} (70 h) is app. 0.720µg/L CYP. Determining the LC_{50} after 24 h was not possible. *D. pulex* was too insensitive to the agent. The same applies to the 7 d experiment. All individual were alive even at the highest concentration after 24 h.

This Cladoceran is much more tolerant towards CYP than *S. vetulus* from the test mentioned above. Compared to biomonitoring data, *S. vetulus* is less sensitive in the lab (3.2). *D. pulex* is even exceeding this high value (approx. 0.5 µg/L CYP) in the biomonitoring study. Thus, when interpreting summarized zooplankton data, one has to keep in mind that different animals can vary widely in their sensitivity towards the agent.

3.4 Water quality parameters

Water quality parameters oxygen content/saturation, pH, alkalinity and conductivity showed no significant deviations from the control range (no significant data ($p<0.05$) for NOEC or PRC). Existing differences at the beginning of the study are due to variations between the enclosures themselves and not due to any toxic effect (data not shown). A summary of these parameters for both years of the study is given in Table 15.

Table 15: Overview table of the water quality parameters in CYP and control enclosures

	O2 [%]		pH		alkalinity [$CO3^{2-}$]		conductivity [µS/cm]	
	CYP-treated	control	CYP-treated	control	CYP-treated	control	CYP-treated	control
mean	98.6	98.1	8.9	9.1	62.4	58.0	137.6	129.2
std. dev.	19.4	19.0	0.5	0.4	10.9	8.3	23.4	15.5
min	60.1	65.5	7.7	8.2	35.2	35.2	90.1	95.8
max	143.7	153.9	10.0	9.9	110.0	88.0	209.0	182.2

Figure 7 depicts the development of the water temperature in the control and CYP enclosures. They range between app.6°C and 22°C. A distinct annual variation can be seen.

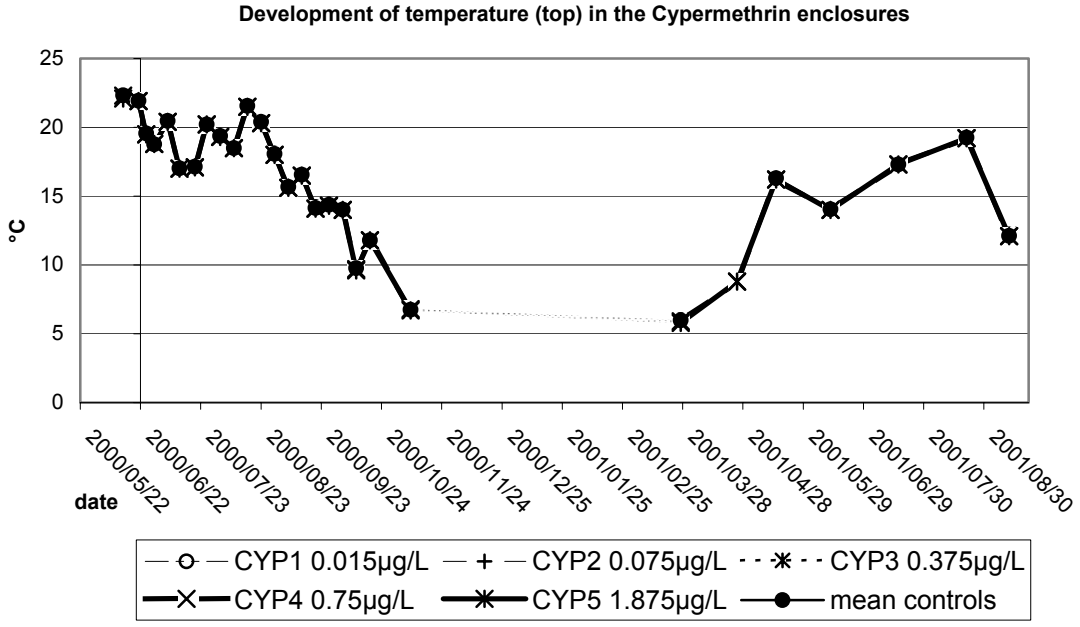


Figure 7: Water temperature in the single substance study

The water chemistry data are not affected by the CYP treatment (no significant data ($p<0.05$) for NOEC or PRC). Table 16 characterizes the CYP and the control enclosures.

Table 16: Summary of water chemistry data in the CYP study

		mean	std. dev.	min	max
TP [µg/L]	CYP-treated	26.89	18.35	8.67	198.76
	control	26.89	18.35	8.67	198.76
SRP [µg/L]	CYP-treated	6.95	8.40	0.00	65.52
	control	10.01	14.40	0.73	139.78
NO3-N [mg/L]	CYP-treated	0.019	0.012	0.006	0.067
	control	0.039	0.063	0.001	0.614
NH4-N [mg/L]	CYP-treated	0.05	0.02	0.01	0.16
	control	0.05	0.02	0.01	0.16
silicate [µg/L]	CYP-treated	235.19	239.06	36.65	933.42
	control	454.95	368.30	29.32	1290.17
Na+ [mg/L]	CYP-treated	2.96	0.37	1.73	4.06
	control	2.96	0.37	1.73	4.06
K+ [mg/L]	CYP-treated	0.06	0.04	0.00	0.22
	control	0.06	0.04	0.00	0.22
Ca2+ [mg/L]	CYP-treated	12.85	4.45	7.81	23.98
	control	12.85	4.45	7.81	23.98
total hardness [°DH]	CYP-treated	3.93	0.54	3.20	5.40
	control	3.93	0.54	3.20	5.40

No effects of CYP on the parameters above were also found by SANDMANN 2000, HUBER *et al.* (unpublished), and GRÜNWALD 2000. This reaction can be excepted for an insecticide (NEUGEBAUR-BÜCHLER, DRAXL *et al.* 1994).

The test system is an oligo-mesotrophic one (SCHWOERBEL 1999).

The content of chlorophyll a is a highly integrating parameter indicating changes in the phytoplankton succession. Figure 8 shows the development of this value plotted against the time before (negative x values) and after the treatment. Since there are a lot factors[2] with greater or smaller impact on this parameter, a lot of noise can be expected (and is observed, indeed). Together with the low nutrient content (Table 16) and the abundant macrophyte growth (Figure 9, page 45), rather small amounts of the pigment are normal. The curves are therefore prone to be over-interpreted.

[2] e.g. temperature, light conditions, nutrients, biocoenosis interaction: competition, zooplankton, macrophytes etc., seasonal development

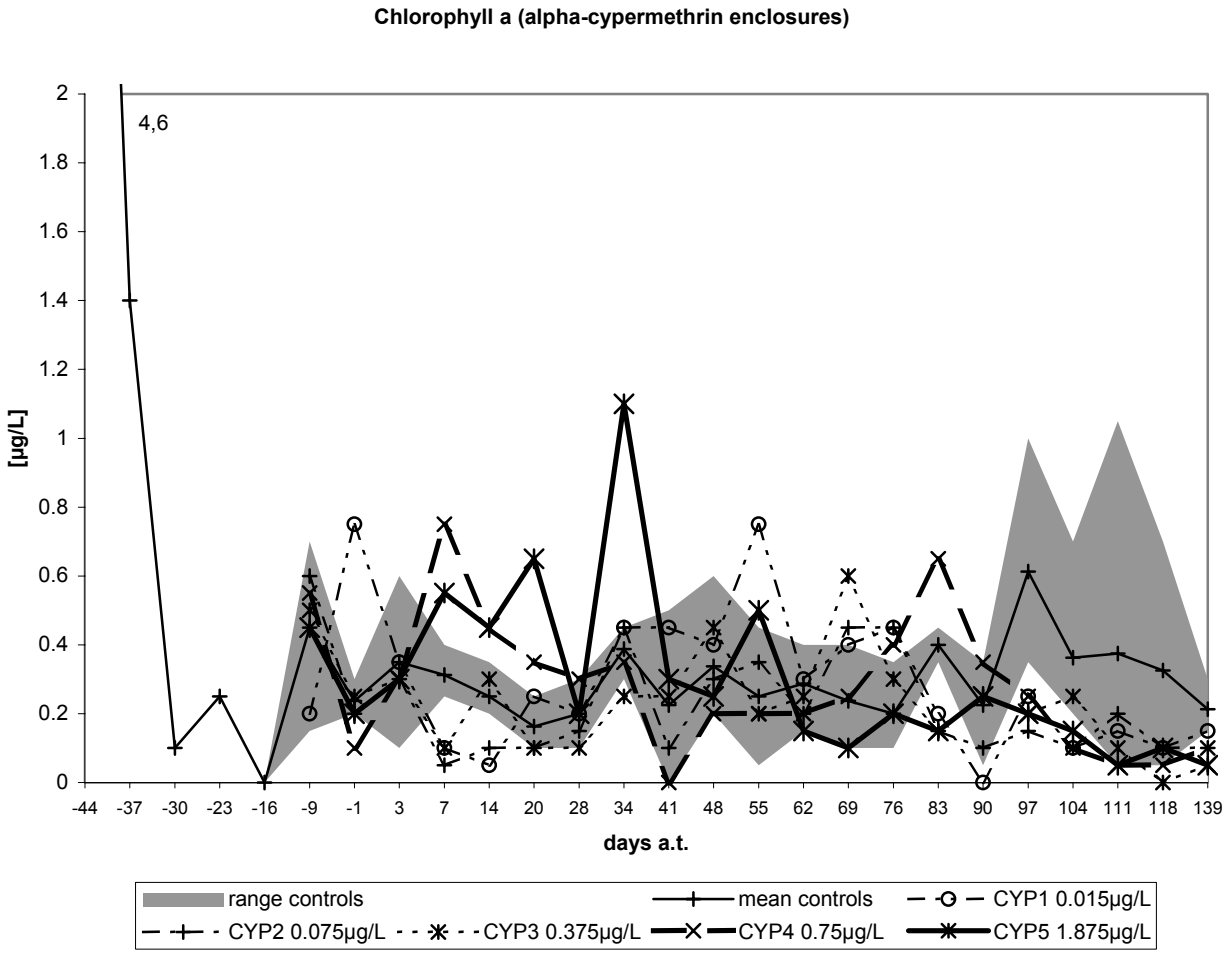


Figure 8: Development of chlorophyll a in the CYP encloures

In spring, before the introduction of the enclosures, chlorophyll a reaches its seasonal maximum with 4.6 µg/L. From day 7 to day 34 a.t., smaller effects of CYP treatment can be seen. The content in the two enclosures with the highest treatments rises above the controls range. The decrease of the CYP1-3 enclosures is too close to the detection limit (about 0.1 µg/L) to really indicate a change due to the agent. The same holds true for the decline to the end of the year (days 83-139). Single peaks are outliers due to the general high noise level and cannot be interpreted. NEC calculation was only possible for day 20. The values are 357.9 µg/L, 443.9 µg/L, and 550.7 µg/L (lower, middle, higher endpoint). An explanation for the pattern is given in 3.6.2. Slightly increased chlorophyll a contents were also found by SANDMANN 2000, for example.

3.5 Macrophytes

Submersed plant cover was not affected by CYP. A general increase is evident (Figure 9). Starting at a level of between 30%-50% covered area, after nearly one year of growth about 70%-90% are reached. Differences between the controls and the treated enclosures were not statistically significant (on each sampling date).

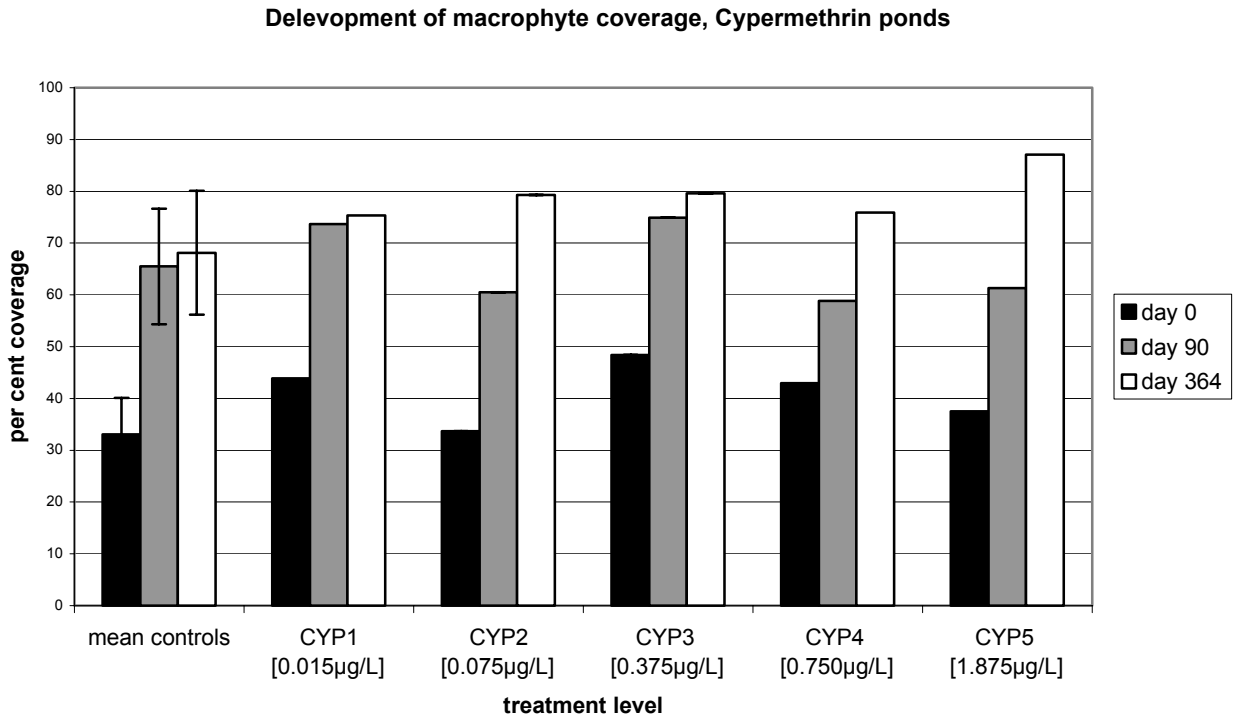


Figure 9: Development of plant cover (%) in CYP enclosures

No effects of Cypermethrin on macrophytes was also found by HILL 1985 and CROSSLAND 1982.

3.6 Phytoplankton

3.6.1 Composition of phytoplankton

The phytoplankton biocoenosis was composed of the classes Bacillariophyceae (8 taxa), Chlorophyceae (49 taxa), Chrysophyceae (15 taxa), Conjugatophyceae (7 taxa), Cryptophyceae (3 taxa), Cyanophyceae (9 taxa), Dinophyceae (2 taxa), Euglenophyceae (4 taxa), Xantophyceae (2 taxa), and one Prasinophyceae.

The predominant taxa of the microscopic analysis are listed in Table 17. The taxa at the first two ranks are both Cryptophyceae, follwed by a Chlorophyceae, a diatom, and yet another Cryptophyceae. *Monosiga varians* is the first Chrysophyceae in this ranking. The phytoplankton community is therefore dominated by Cryptophyceae. Only the taxa of the Cryptophyceae mentioned in this "top ten" list score 59.5% dominance.

Table 17: Dominant taxa in CYP phytoplankton

rank	species	dominance (CYP), %
1	*Chroomonas acuta* (Cryptophyceae)	30.1
2	*Cryptomonas erosa/ovata* (Cryptophyceae)	26.7
3	*Nephroselmis olivacea* (Chlorophyceae)	5.3
4	*Achnanthes minutissima* (Bacillariophyceae)	3.9
5	*Katablepharis ovalis* (Cryptophyceae)	2.7
6	*Monosiga varians* (Chrysophyceae)	2.5
7	Chlorophyceae *ssp.*	2.4
8	*Desmarella moniliformis* (Chrysophyceae)	2.4
9	*Mallomonas sp.* (Chrysophyceae)	1.9
10	Cyanophyceae	1.8

Distributions of classes against time in the controls and the highest treatment level are shown in Figure 10 and Figure 11, respectively.

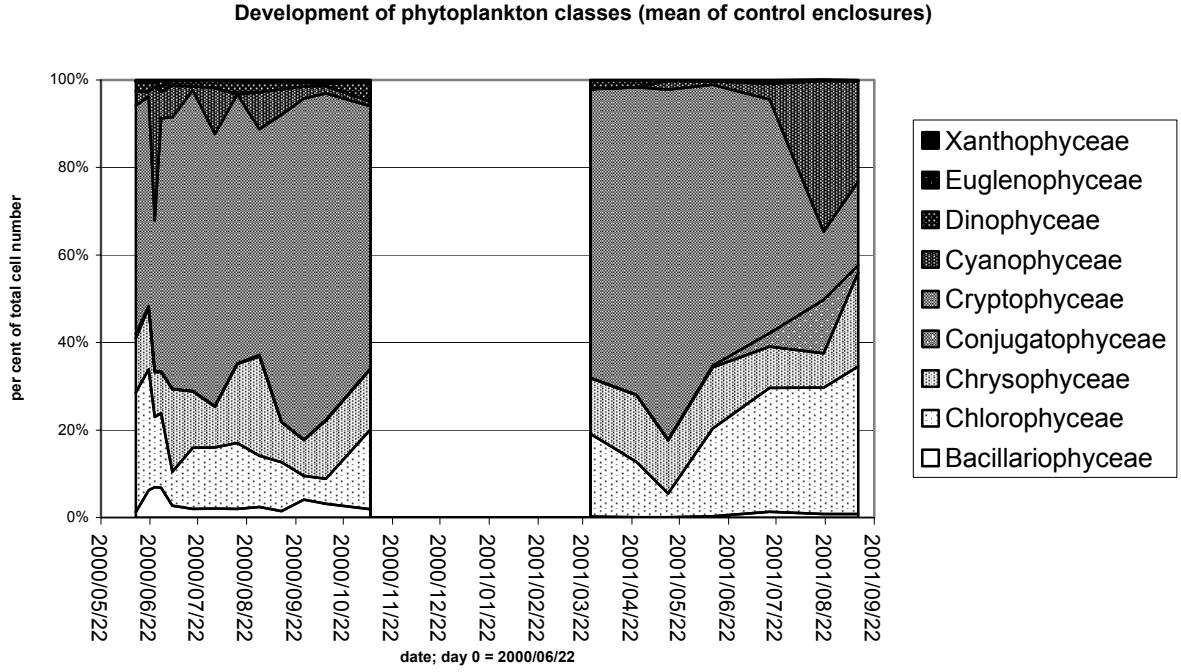


Figure 10: Abundance data by phytoplankton class distribution in the controls of the single substance studies

A slight disturbance by the sampling shortly after day 0 of the study is revealed (Figure 10). This is due to a temporary decrease in Cryptophyceae that is leveled out quite fast.. Algae of different classes are distributed rather constantly in the controls except for a major decrease of Cryptophyceae in fall 2001. Chlorophyceae and Cyanophyceae, as well as Conjugatophyceae, took advantage of that.

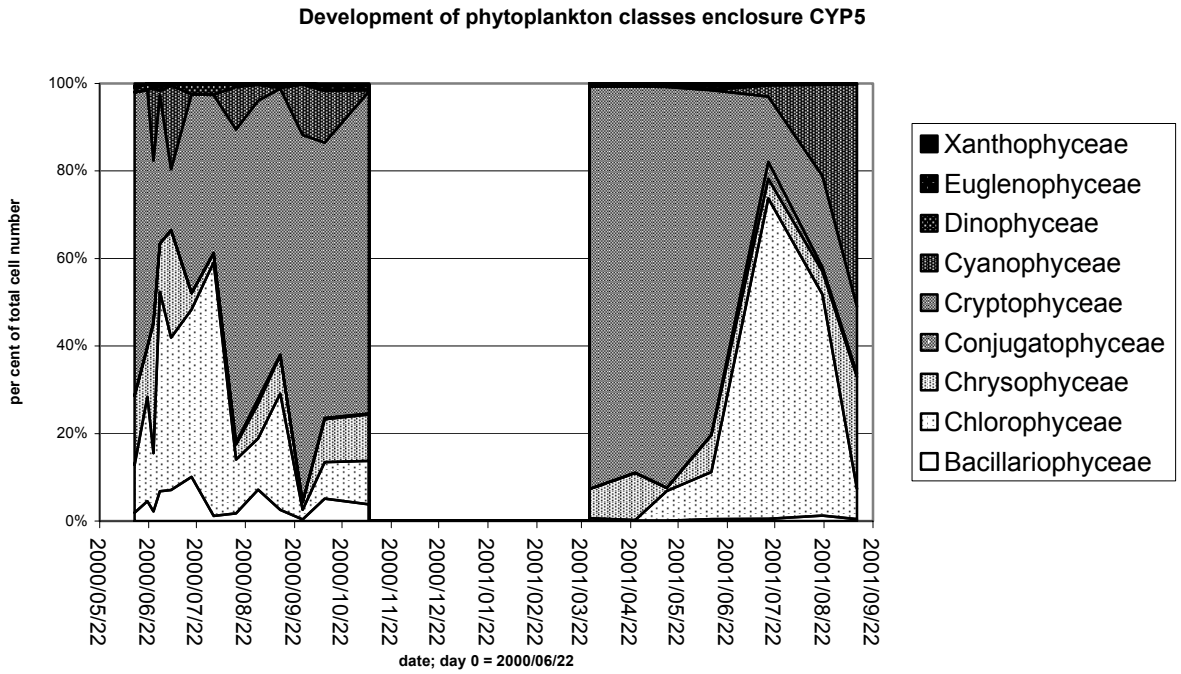


Figure 11: Abundance data by phytoplankton class distribution in the CYP5 (1.875 µg/L a.i.) enclosure

Dispersal of classes in the CYP5 enclosure (Figure 11) indicates a short-term impact on the system. Cryptophyceae are reduced but can compensate for that in about two months' time. Mainly Chlorophyceae and Chrysophyceae can profit in that time slot. Lower treatment levels follow that pattern but with ever lower peaks.

By mid-summer 2001 Chlorophyceae reach a maximum. This is more or less a "special feature" of this enclosure and cannot be observed in the other treated ones (data not shown). At the same time, Cryptophyceae begin to decline (like in the controls, see Figure 10). This may be due to the "normal" development of the system as it can also be seen in all other enclosures. CYP is surely degraded completely by that time (3.1) and there is no indication for a secondary effect of the agent as a reason for this. It is more likely that the higher macrophyte density that is present in all enclosures in 2001 compared to 2000 (3.5) leads to such a process. When the submersed plants start dying in autumn, changes in light and nutrient conditions are inevitable. In the first year of the study, plant cover is less and so these effects may not be enough to change the algal composition. The increase in Cyanophyceae, often found as the "winner" when more nutrients enter the water (LAMPERT and SOMMER 1993), backs this assumption.

3.6.2 Abundance data

A selection of taxa abundance data is presented here. These are some with a typical reaction to the treatment or "important" ones as shown in dominance and/or PRC analysis.

3.6.2a Total abundance

Phytoplankton abundance was at about 1000 cells per litre with a decrease to the later days (Figure 12). In the second year of the study, no effects were apparent.

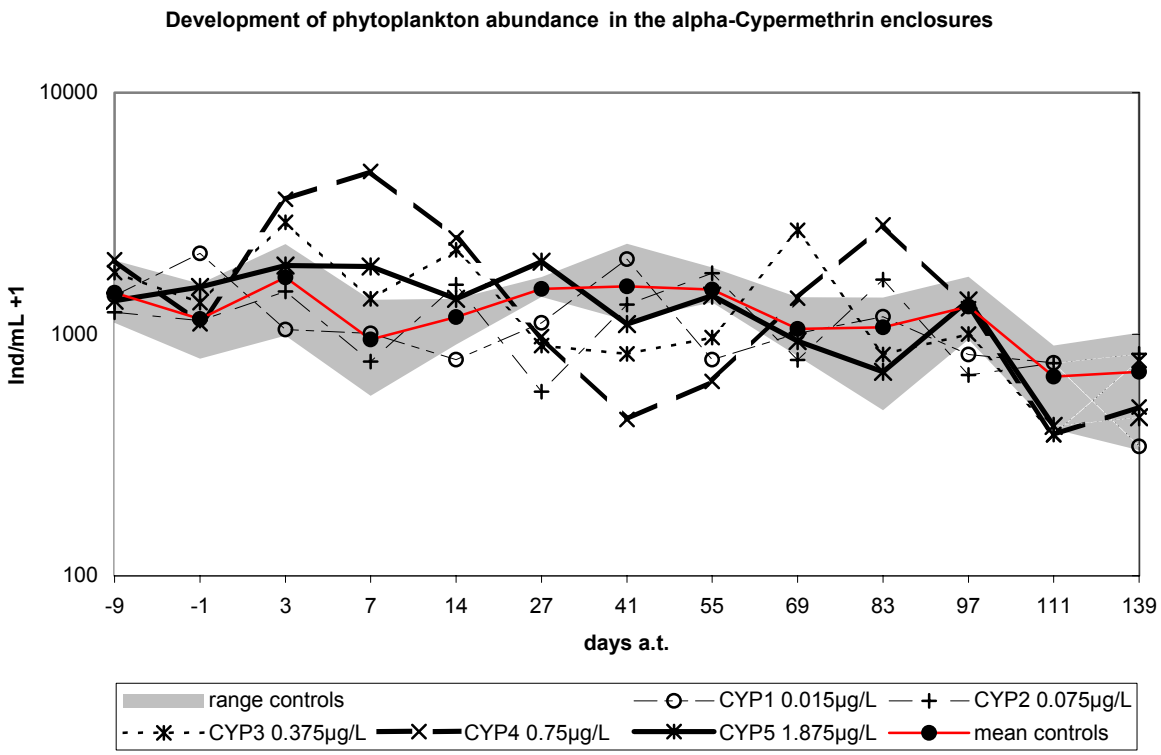


Figure 12: Phytoplankton abundance in the CYP study

The biggest variation can be seen in enclosure CYP4. More algae were found on day 3 to 14 and on day 83 a.t., less from day 27 to 55 a.t.. CYP3 shows a more or less similar development with lower maximums. The highest treatment level lay within the control range most of the time and did not exceed it too far. The two lower CYP levels reveal no effect. So apparently the algal abundance does not follow a linear pattern with CYP concentration.

It can be explained by secondary effects in combination with a slight direct toxicity. The insecticide reduces grazers (see 3.7 Zooplankton, especially *S. vetulus*) in the first three weeks following the application. So more algae can be present in the system in that time slot. Additionally, algae might be negatively affected in CYP5. Abundance is lower than in CYP4 (three times) or CYP3 (twice), although the higher the enclosures are treated with CYP the less grazers can be found (3.7.2). The effect of the lowered number of grazers being the only influence on the system cannot explain this. Moreover, it is rather opposed to it. Assuming a minor toxicity of CYP towards algae, however, does it.

Comparable early effects can be seen in Chlorophyceae (Figure 18), Chrysophyceae (Figure 19) and cryptohyceae (Figure 14 and Figure 15).

After the first month since treatment, and while the very sensitive zooplankton predator *Ch. crystallinus* is still not in line with the controls, grazers can over-compensate for the loss (being released from top-down control, see 3.7.2) and eventually reduce algal abundance. This is supposed to be a graded effect with concentration of the agent, leading to less algae the higher the treatment level has been. This interpretation does not readily fit with the fact that the highest treatment level does not show the biggest variation. So here, CYP residues seem to be high enough to limit grazing by hindering grazing effectiveness (DAY and KAUSHIK 1987,

FERNANDEZ-CASALDERREY, FERRANDO *et al.* 1994). The indicated minor direct effect could support such an effect, too.

NOEC calculations revealed no effect up to an amount of 0.075 µg/L a.i.. Calculations were significant ($p<0.05$) for day 3 a.t. to day 14 a.t.. No constant NOEC on two consecutive dates could be calculated for the later consequences of the treatment.

There were no effects in the second year of the study.

3.6.2b Species richness

Species richness is an important end point for ecotoxicological studies. Shifts here affect ecosystem structure. Maintaining biodiversity is one of the major topics in nature conservation (EU, 2002). Figure 13 illustrates the situation in the CYP study.

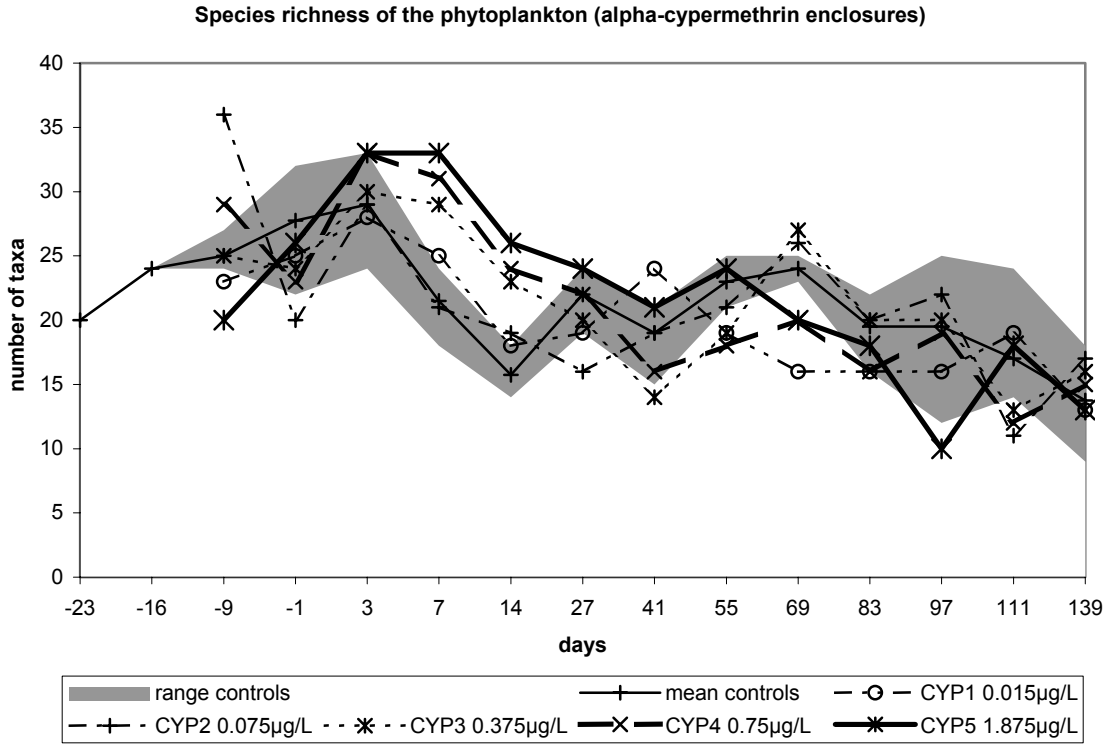


Figure 13: Species richness of the phytoplankton in the CYP enclosures

As presented here, even more species can be found in the treated enclosures in the two weeks following the treatment (ideally graded with amount of a.i.). Then numbers of taxa are in line with the controls again (minor changes excluded). This increase is due to the reduced grazing (explanation given in 3.6.2a; cf. also 3.7.2). It is implausible that new species arrive in the system in such a short time. More probably, species with an abundance below the detection limit of the microscopical analysis are then able to grow to large enough numbers to be "found". NOEC is either 0.075 µg/L (day 7) or lower than CYP1 (day 14), so it does not meet the requirements for a definite rating. NEC is between 0.020 µg/L and 0.078 µg/L (these two dates). Giving a rather restrictive interpretation, a no effect level may be the 0.015 µg/L as in CYP1.

3.6.2c Chroomonas acuta

The distribution with time and treatment level of this algae is given in Figure 14. It is in agreement with the trends and explanations in the total abundance: In the first month there is an increase and then a decrease. The effect is strongest in CYP4; a rationale for the lowered impact in CYP5 is given in 3.6.2a. Effects are less pronounced than in the total phytoplankton abundance. There is not such a high increase or decrease. Counts rising above the control range from day 69-97 a.t. may indicate a general, unspecific disturbance of the system due to the treatment. Since a definite reason cannot be given, it is probably better to see this effect as a "normal" fluctuation of the system on its path towards the untreated state.

NOEC for this taxon is 0.375 µg/L.

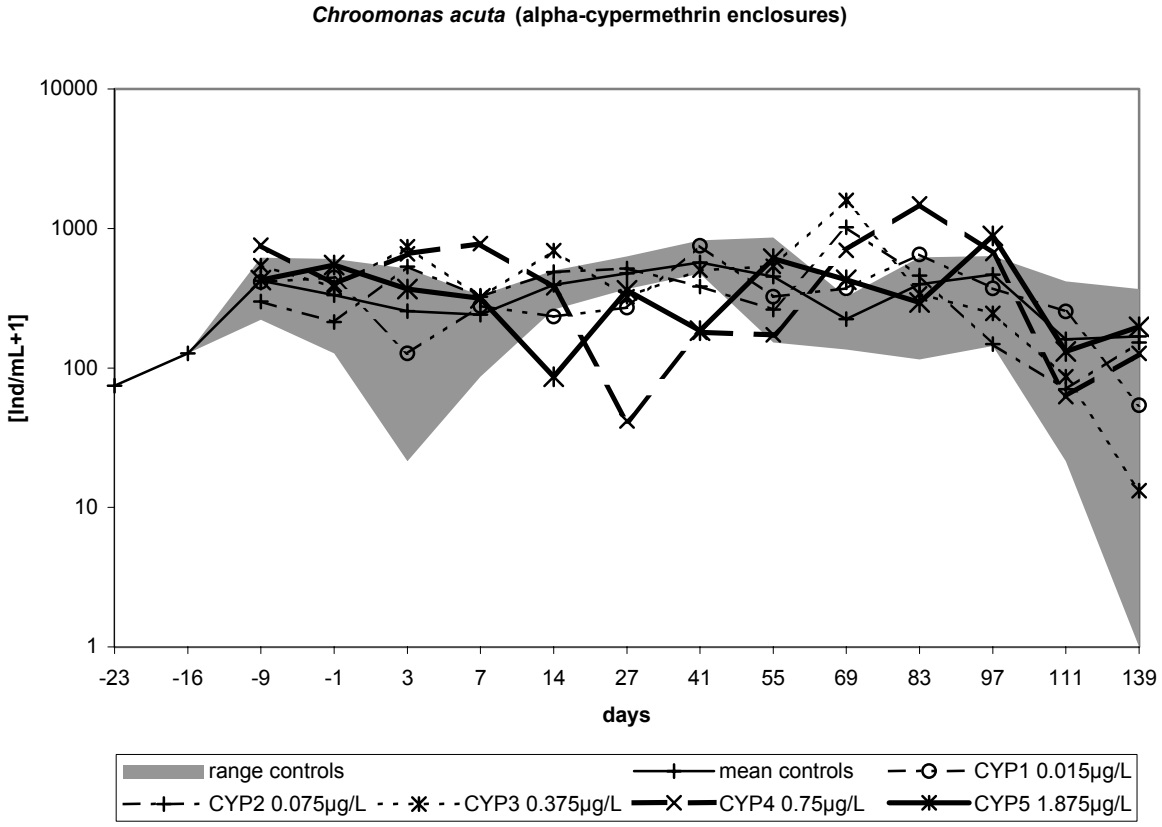


Figure 14: Development of *Ch. acuta* in the CYP enclosures

3.6.2d Cryptomonas erosa et ovata

Here we have to separate between the two years of the study. In the year of the application (Figure 15), there is a very much attenuated reaction similar to the one the total abundance reveals (Figure 12): A minor increase in the first month a.t. followed by a decrease. Again, impact is strongest in CYP3 and CYP4. CYP5 does not show the increase but only the decrease. As noted above, these effects can be addressed to secondary food web interactions and a minor direct toxicity. A progession that is not closely related to the treatment level, like it is found here, is no surprise for such an indirect impact.

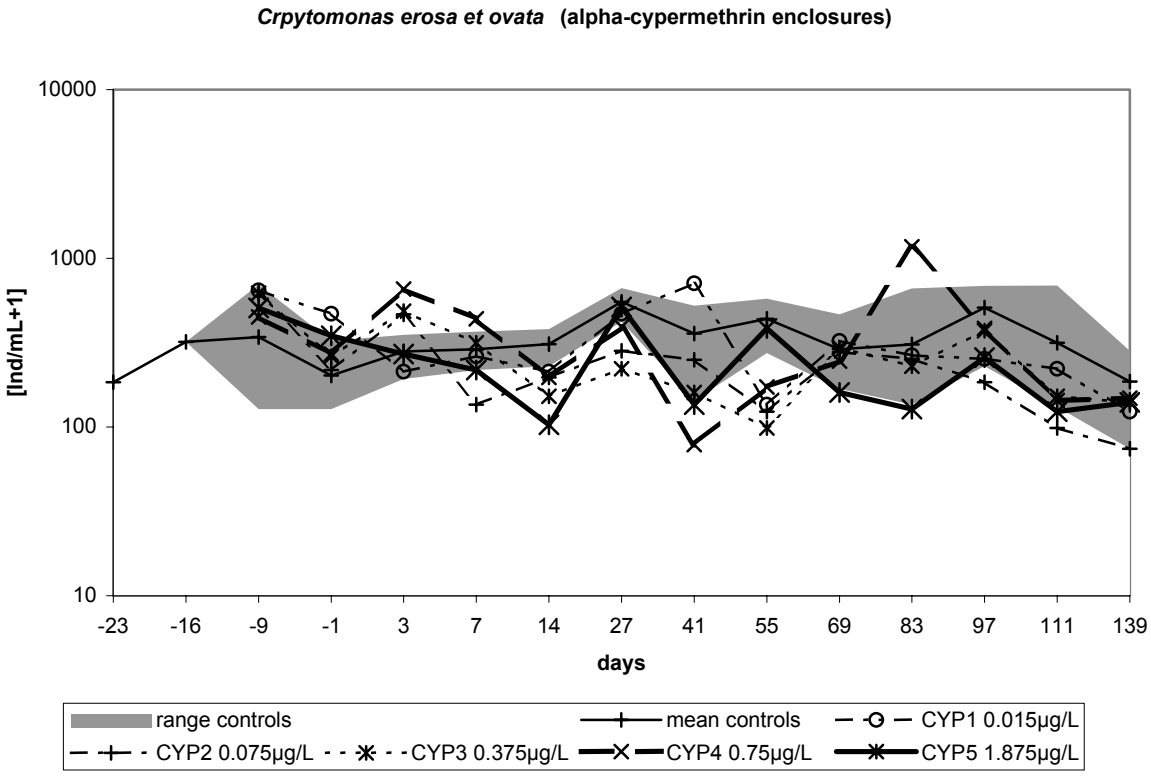


Figure 15: Development of *Cr. erosa et ovata* in the CYP enclosures (first year)

All in all, there is not too much deviation from the control range. This taxon has been present in high numbers with little variance. Subsequently, the Williams' test gets quite sensitive. The NOEC is 0.015 µg/L for the first year, every time indicating a negative trend with concentration. *Cr. erosa et ovata* shows mainly the later secondary effects and merely a minor increase in the first weeks. The slight increases in CYP4 on day 3 and 7 a.t. could not be confirmed by the statistics. There may be two reasons for a mere "late"secondary reaction:

1. Generation time of this algae is too long to really gain from the cut-down in grazing.
2. The taxon is the main nutrition[3] for grazers and not released from top-down control.

In the second year (Figure 16), abundances are lowered in the higher treatment levels (but not in a treatment related order). Since there are no pesticide residues to be found any more, a direct impact must be excluded.

Higher numbers in zooplankton grazers (see 3.7.2) due to reduced predation are a possible reason for this development. This holds true for CYP5 at least. The development in CYP2 cannot be explained readily and may also be a chance effect, because treatment impact had not been too intensive at all. - The other enclosures are quite near the controls, so the relation may be blurred to some extend. NOEC in this year is 0.015 µg/L a.i..

Cryptophyceae react in almost the same way and are not shown separately.

[3] In fact INFANTE 1973 and AHLGREN 1990 found *Cr. ovata* to be an important nutrition for zooplankton

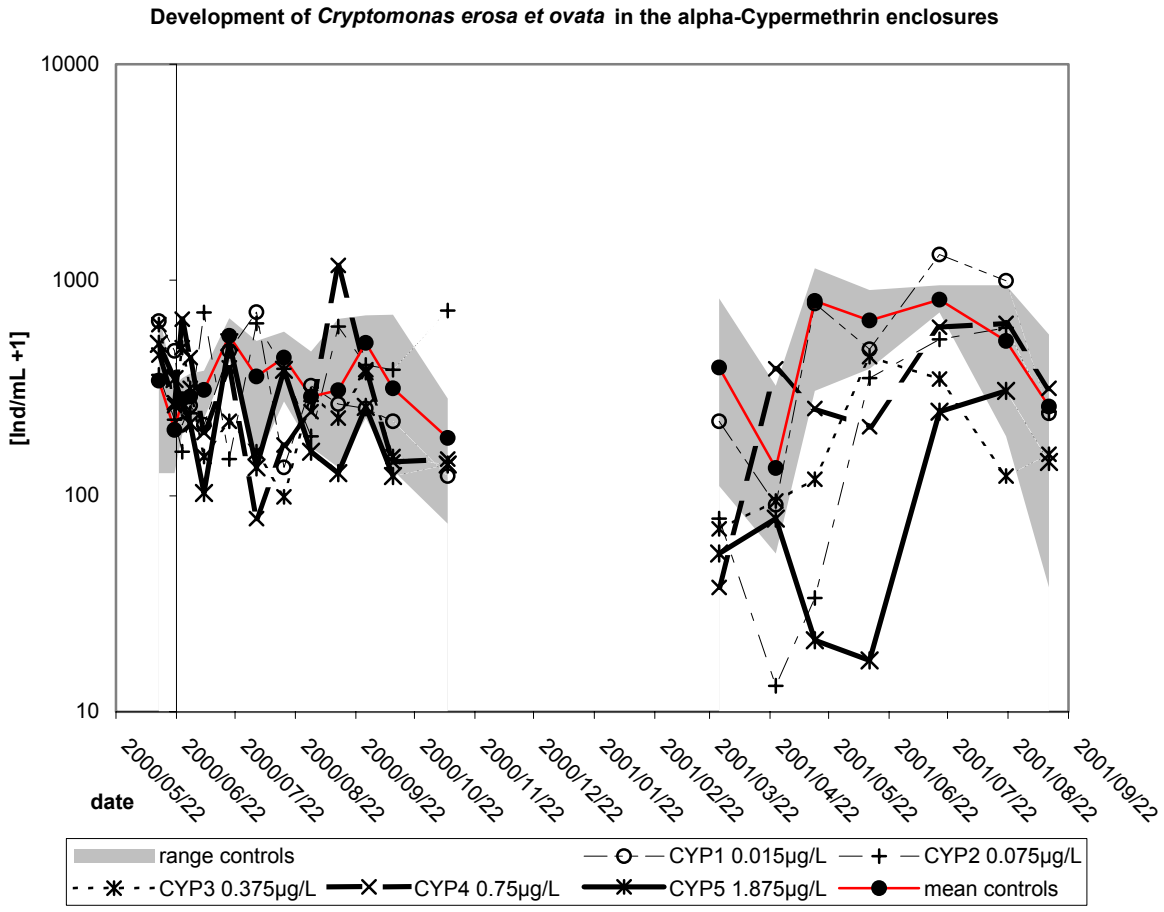


Figure 16: Development of *Cr.erosa et ovata* in the CYP enclosures (2 years)

3.6.2e Bacillariophyceae

This class has lower abundances than the others presented here (Figure 17). The control range indicates that sometimes no cells at all could be found. Indeed, there is a lot of noise in the data that is complicating interpretation.

NOEC and NEC calculations did not lead to convincing result. Still, what must be noted are the rising counts in the two highest treatment levels short time after the application (day 3 to 27 a.t.). This may be due to reduced zooplankton abundance.

From day 55 to 69 a.t. there is an increase in all but the lowest treated enclosures. Highest scores are for CYP3, the others are more or less the same. This unimodal reaction is indicating yet another secondary effect. It may be due to selective feeding of *Eu. gracilis* (INFANTE 1973); a species that was present in the enclosures in question in high abundances during that time (Figure 28, page 66). The Bacillariophyceae may not be fed on so extensively and can therefore profit from the increased grazing pressure on the other algae. With the system getting back to "normal" this effect vanishes as well (day 83 a.t. and later).

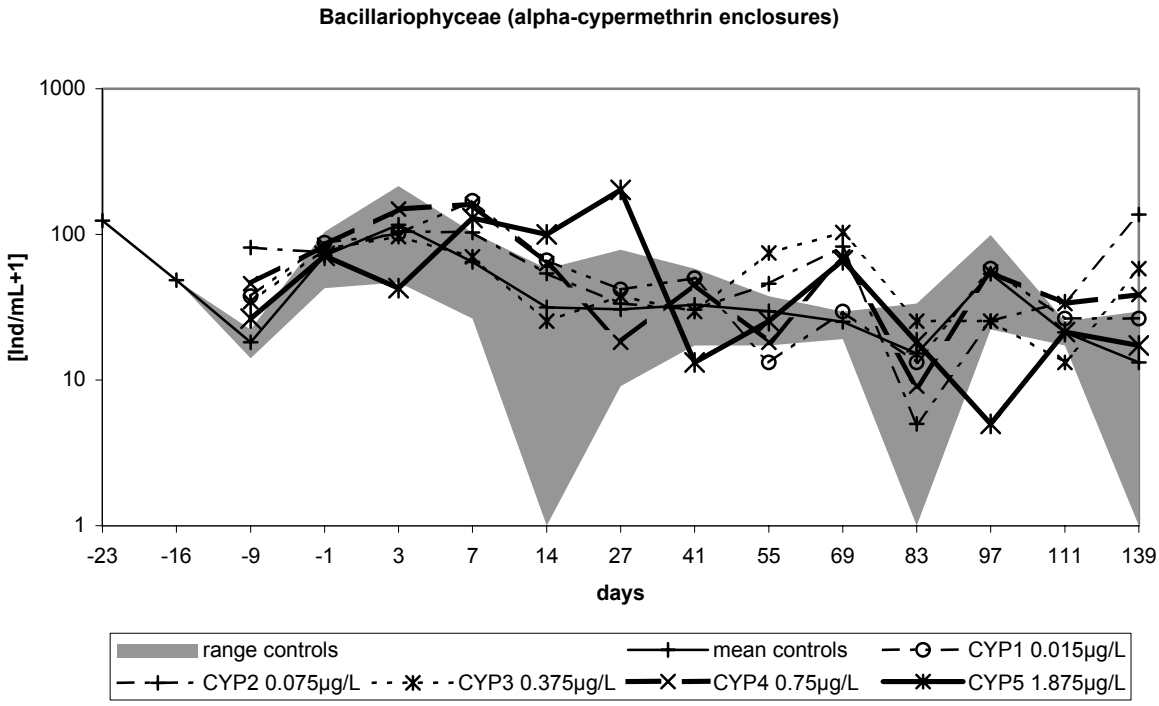


Figure 17: Development of Bacillariophyceae in the CYP enclosures

3.6.2f Chlorophyceae

Chlorophyceae can profit from the reduced grazing in the three weeks following the application (Figure 18). No later secondary effects can be stated. NOEC is 0.015 µg/L. Here again a minor direct action of CYP may be present, because CYP5 is lower than CYP4.

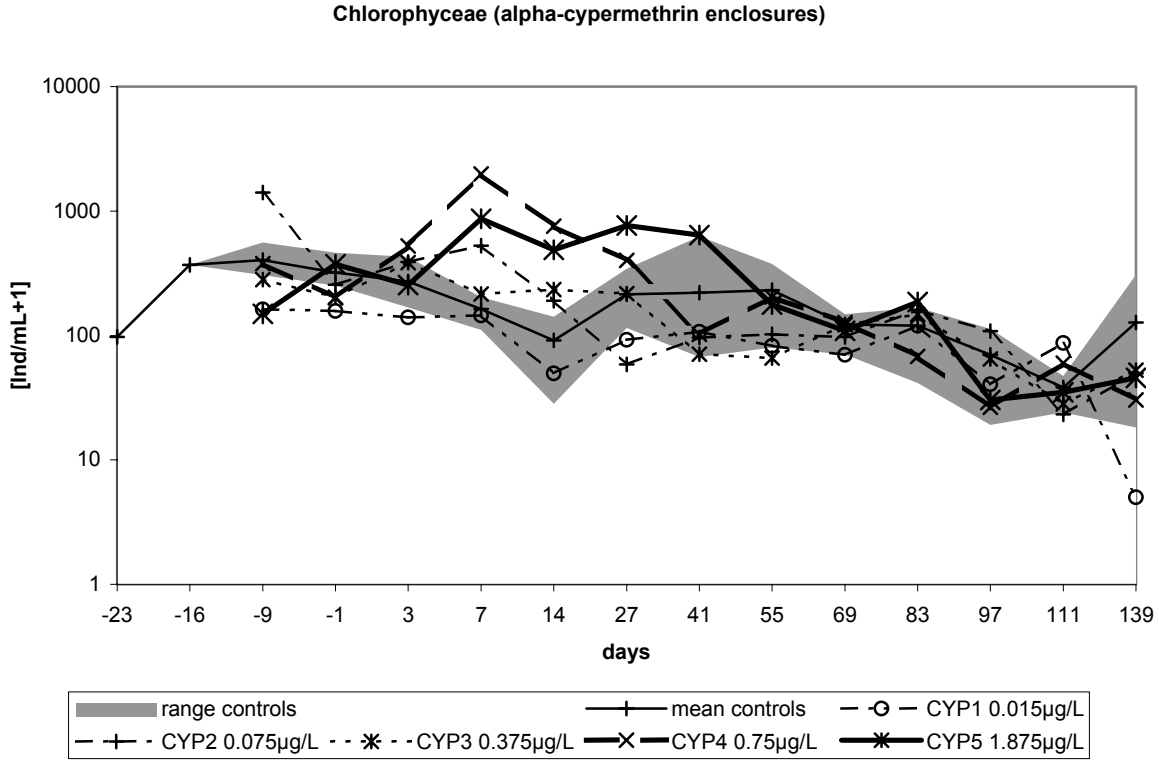


Figure 18: Development of Chlorophyceae in the CYP enclosures

3.6.2g Chrysophyceae

This algal class responds in almost exactly the same way as the total number of algae does (Figure 19 and Figure 12): An increase (day 3 to 14) is followed by a decrease (day 27 to 69). For a rationale please refer to 3.6.2a.

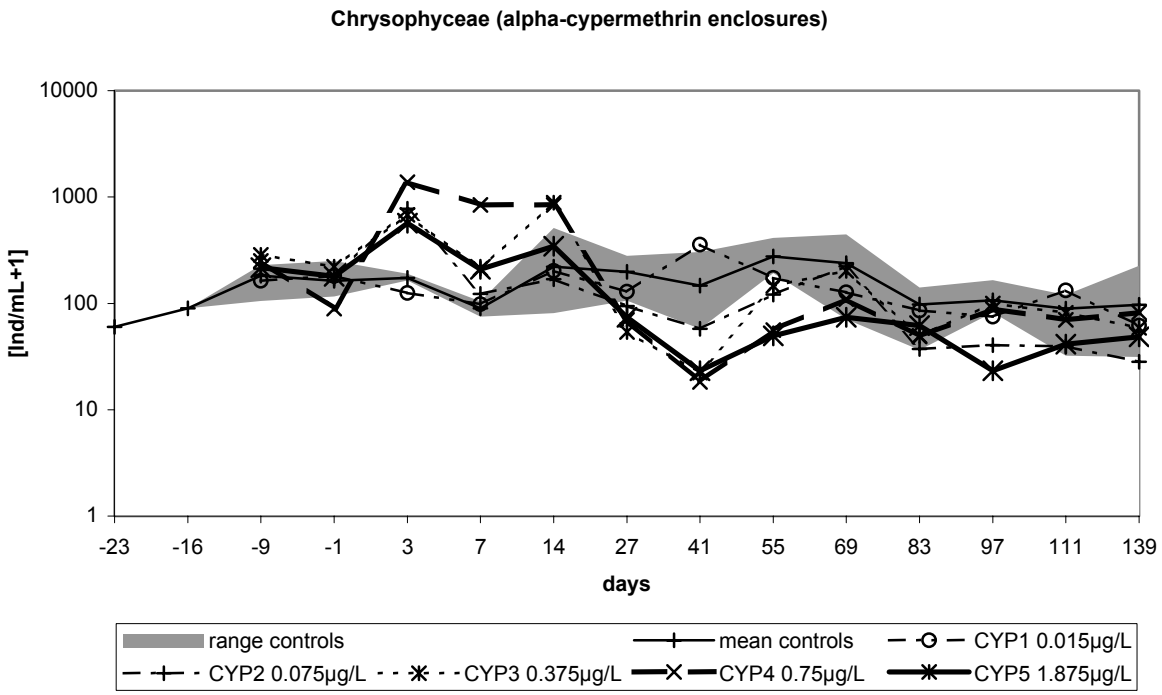


Figure 19: Development of Chrysophyceae in the CYP enclosures

Two different NOEC are possible: 0.075 µg/L a.i. for the increase, 0.015 µg/L for the decrease. The lower one must be taken into account. These findings are corroborated by results of HUBER *et al.* (unpublished) who found a NOEC of 0.075 µg/L and minor direct toxicity as well.

3.6.3 Community analysis

3.6.3a Shannon index and evenness

The evenness of the phytoplankton is presented in Figure 20. PFADENHAUER 1997 stated that the evenness is more appropriate when samples with different species numbers are compared. Means for the evenness are 0.66 for both the controls and the treated enclosures. Shannon index shows almost exactly the same picture and is thus not shown. The Shannon's theoretical H_{max} was 3.55 in this study; mean value in the CYP enclosures is 2.02, in the controls 2.03.

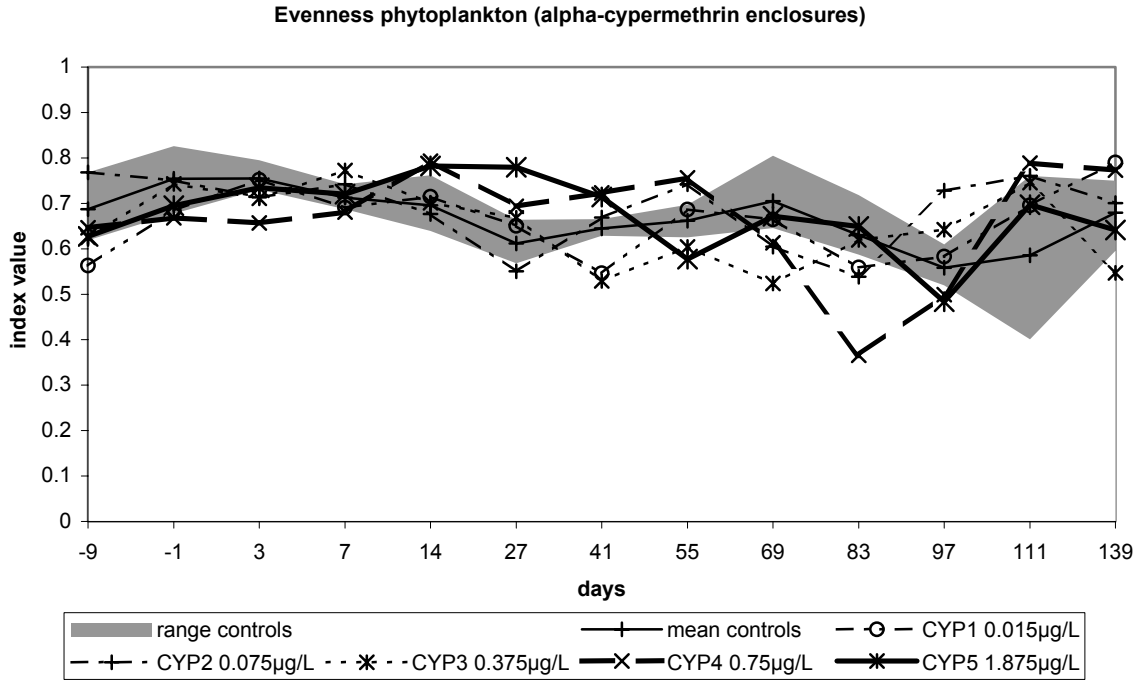


Figure 20: Evenness in the phytoplankton (CYP study)

The progression is on the one hand somewhat comparable to the total abundance figure (Figure 12): More algae in the beginning (day 7-41), less afterwards (up to day 83). Explanation can be given in the same way: Both are secondary effects stemming from divergent grazing pressure of the zooplankton. Note that CYP5 is the treatment level with the biggest deviation from control (short-term effect).

On the other hand, the index focuses on diversity, so it is not surprising that the pattern is also analogous to the species richness (Figure 13).

Derived from these curves, no effects occured up to CYP2 (0.075 µg/L). Mean NEC values were all too high to be sensible. The lowest NEC is 0.186 µg/L CYP for the Shannon index and 0.100 µg/L for the evenness.

3.6.3b RAD index

RAD index tells us about the "distance" between two groups (i.e. sets of enclosure data), so it is a measure of dissimilarity (maximum value=2). Here, the phytoplankton community was entered in the analysis. Treated ponds were compared with the controls. Figure 21 shows that the system has completely recovered from the treatment until day 97 a.t. (regarding only the first year; up to day 139 a.t.). Major deviations are found in the three highest treatment levels, NEC is 0.296 µg/L (mean lower value of the first year, the others were too high to be reasonable, n=2). All other enclosures have a more or less similar algal biocoenosis. Since the RAD does not tell us anything about an increase or decrease in abundance or species richness directly, one can only state that recovery takes place within a little more than three months after the application (data up to day 139 a.t.).

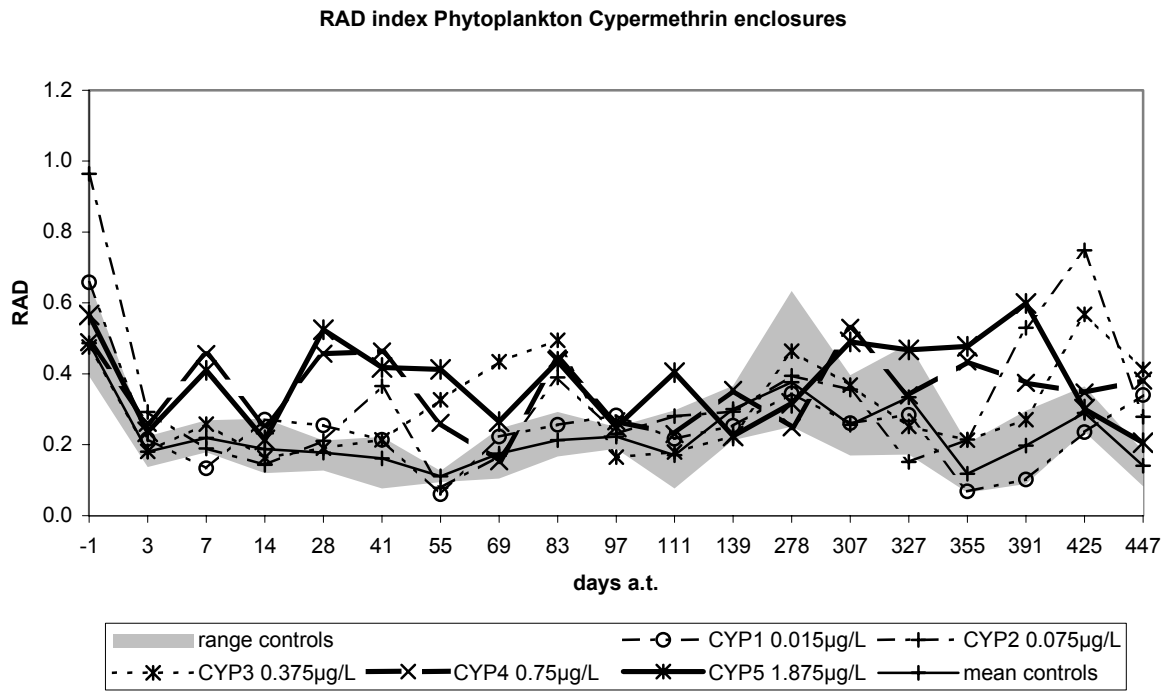


Figure 21: RAD index of phytoplankton (CYP study)

In the second year of the study (first sampling on day 278), the communities get dissimilar to each other again, when the invertebrate predator *Chaoborus crystallinus* is in line with the controls again (Figure 25, page 63). The two highest treated enclosures are above the control range on day 307 a.t. already, CYP2 and CYP3 follow later in the year. The highest deviation is seen in CYP2 on day 425 a.t.. Hence, the recovery of the main zooplankton predator imposes an impact quite as high as the insecticide treatment (RAD reaches about 0.6). A late secondary effect on the system has to be noted. The effects starts earlier at higher treatment levels. This may be due to the greater disturbance of the zooplankton community when its predator gains in abundance again. In the lower treated levels, at least some *Ch. crystallinus* survived the treatment. A mere increasing abundance is not enough to impose an impact on the algae. So the impact on the algae is delayed until the larvae reach their maximum. No observed effect level is CYP1 (0.015 µg/L).

3.6.3c PRC analysis

Figure 22 shows the development of the cdt values from the PRC analysis on phytoplankton abundance data. CYP3 to CYP5 show major deviations between day 3 and 28 a.t. Interestingly, CYP5 is not the most intensively changed enclosure. After day 28 a.t., the system reaction changes in the opposite direction. Effects are not too pronounced.

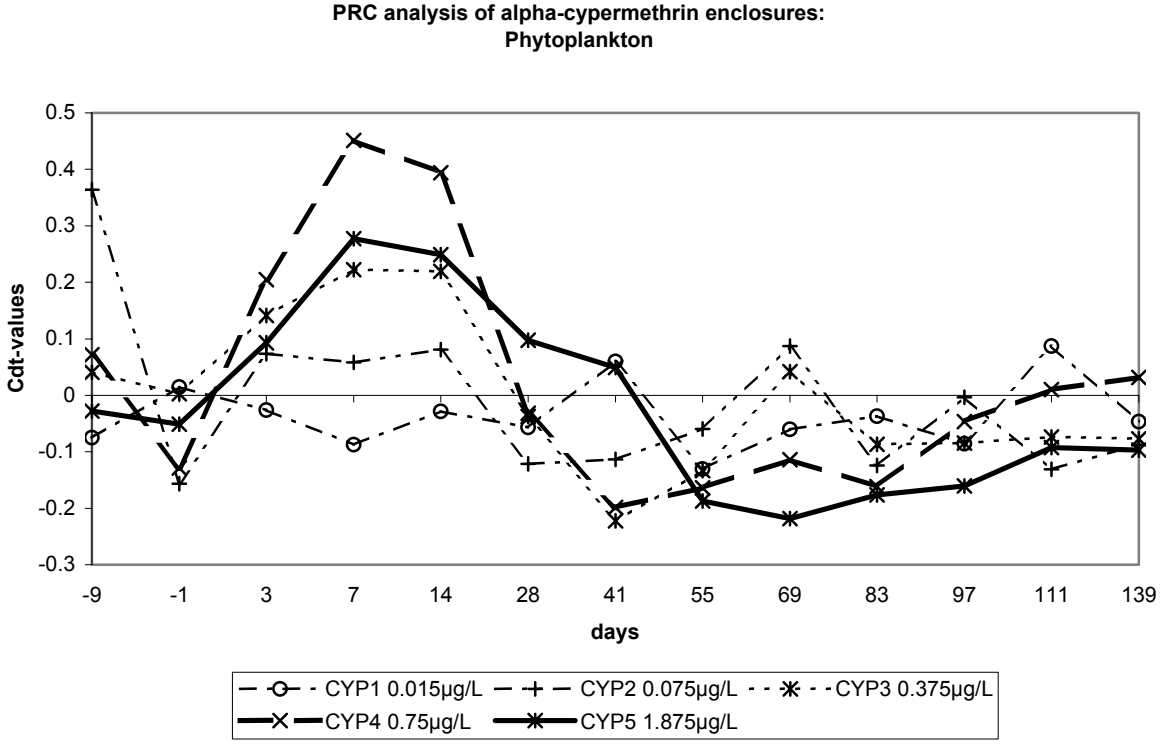


Figure 22: PRC analysis on phytoplankton in the CYP study

This analysis was not significant ($p>0.05$). The main reason for this may be the low direct toxicity stated above. By this process, no linear[4] but a unimodal model is the basis of the reaction to CYP. Moreover, two secondary effects lie underneath the system reaction (cf. 3.6.2a). So for PRC, the data does not meet the assumption of a continuous dose-response model and there is not one treatment triggering the effects directly.

Analysis was therefore limited to the data up to day 55, taking mainly the first effect into account. This PRC is significant ($p=0.015$) and explored in further detail. The treatment explains 32.7% of the variance. Species scores (all taxa with an absolute value >0.5) are given in Table 18.

Table 18: Species scores of the restricted PRC on phytoplankton

Taxon	Species Score
Coelastrum sp. (Chlorophyceae)	0.69
Monosiga varians (Chrysophyceae)	0.66
Nephroslemis olivacea (Chlorophyceae)	0.57
Desmarella moniliformis (Chrysophyceae)	0.53
Gomphosphaeridideae (Cyanophyceae)	0.53

PRC curves of this restricted analysis are almost the same as the ones in Figure 22 up to day 55 a.t. and are therefore not shown separately. The $NOEC_{community}$ is 0.075µg/L, derived from Williams' tests.

[4] as expected by PRC analysis (TER BRAAK and VAN DEN BRINK 1998, 1999)

3.6.4 Overview of treatment effects of CYP on phytoplankton

There were mostly short-time effect of CYP on phytoplankton. In the second year of the study, no direct effect of the treatment could be observed. A summary of NOECs from Williams' tests for some taxa is given in Table 19. Please note that NOECs are only considered valid ($p<0.05$) if they could be calculated on at least two consecutive dates.

Table 19: NOEC summary phytoplankton (CYP study)

taxon	NOEC [µg/L]	direction of CYP influence on data /remarks
Chlorophyceae	0.015	up
Chroomonas acuta (Cryptophyceae)	0.375	up, later slightly down
Chrysophyceae	0.015 for decrease	secondary effects: at the beginning up, then down
Cryptomonas erosa et ovata (Cryptophyceae)	0.015	fluctuating pattern in the first year, decrease in the second (5 samples)
Cyanophyceae	0.75	up
Desmarella moniliformis (Chrysophyceae)	0.075	weak secondary effects
Monosiga varians (Chrysophyceae)	0.375	secondary effects!
Nephroselmis olivacea (Chlorophyceae)	0.375	up
total abundance	0.075	up, when CYP is present, then down
$NOEC_{community}$	0.075	increasing abundance; but analysis was time-restricted

The lowest value is 0.015 µg/L a.i.. During the time CYP is present in the enclosures, zooplankton abundance is reduced (see 3.7.2, especially *Simocephalus vetulus*). Thus, less grazing is imposed on phytoplankton[5] and it can grow to higher abundance except for CYP5 on some occasions. As a consequence, a minor direct toxicity of CYP on the algae is proposed. Later on, about one month after treatment, when the top predator on zooplankton, *Chaoborus crystallinus* (LIAR 1990), is still negatively affected (see 3.7.4, page 74), grazers like *Eudiaptomus gracilis* increase (see 3.7.2 for details). Algae are then negatively affected by increased grazing pressure[6]. This pattern is especially important for *C. erosa et ovata*, for which a decrease beginning in the 0.015 µg/L enclosure is still present in the second year of the study. Therefore, this algae seems to be the most important nutrition for zooplankton grazers. Long-term loss of the top predator on grazers may enhance the secondary negative effects on the algae and lead to the very low NOEC (0.015 µg/L CYP) in the second year. Because *C. erosa et ovata* is the second most dominate species (Table 17) and effects are still visible in the

[5] This effect is even intensified by reduction of the filtration rates of zooplankton as a sublethal effect of pyrethroids (DAY and KAUSHIK 1987)

[6] Support for this interpretation is found in LIAR 1990, who found algal blooms when invertebrate predation on zooplankton is increasing. Here, the system reacts the other way around.

year following the application, the NOEC of this species is regarded crucial for the system toxicity assessment.

RAD and evenness/Shannon index were less sensitive in detecting effects. RAD reveals recovery within 14 weeks and a secondary effect of the treatment in the following year.

In short, a minor direct toxicity of CYP on phytoplankton at 1.875 µg/L could be observed. Secondary effects related to changes in the zooplankton community lead to an over-all NOEC of 0.015 µg/L CYP for the phytoplankton. This value is backed by the analysis of the species richness indicating no effect at the same amount of a.i..

3.7 Zooplankton

3.7.1 Composition of Zooplankton

The zooplankton community could be divided in 33 taxa: 9 taxa of Cladocera, 3 of Copepoda (including Nauplius larvae), Ostracods, 19 Rotifers and one insect larvae (Diptera), *Chaoborus crystallinus*.

Most dominant taxa are listed in Table 20.

Table 20: Dominant zooplankton taxa in the CYP study

rank	taxon	dominance (CYP), %
1	Cycopoida (Copepoda)	31.4
2	*Simocephalus vetulus* (Cladocera)	17.0
3	Nauplia ssp. (Copepoda)	11.7
4	*Mytilina mucronata* (Rotifera)	10.7
5	*Alona guttata* (Cladocera)	4.6
6	*Eudiaptomus gracilis* (Copepoda)	4.0
7	*Lecane* forma "monostyla" (Rotifera)	3.6
8	*Chaoborus crystallinus* (Insecta)	3.3
9	*Chydorus sphaericus* (Cladocera)	2.5
10	*Polyarthra vulgaris* agg. (Rotifera)	2.4

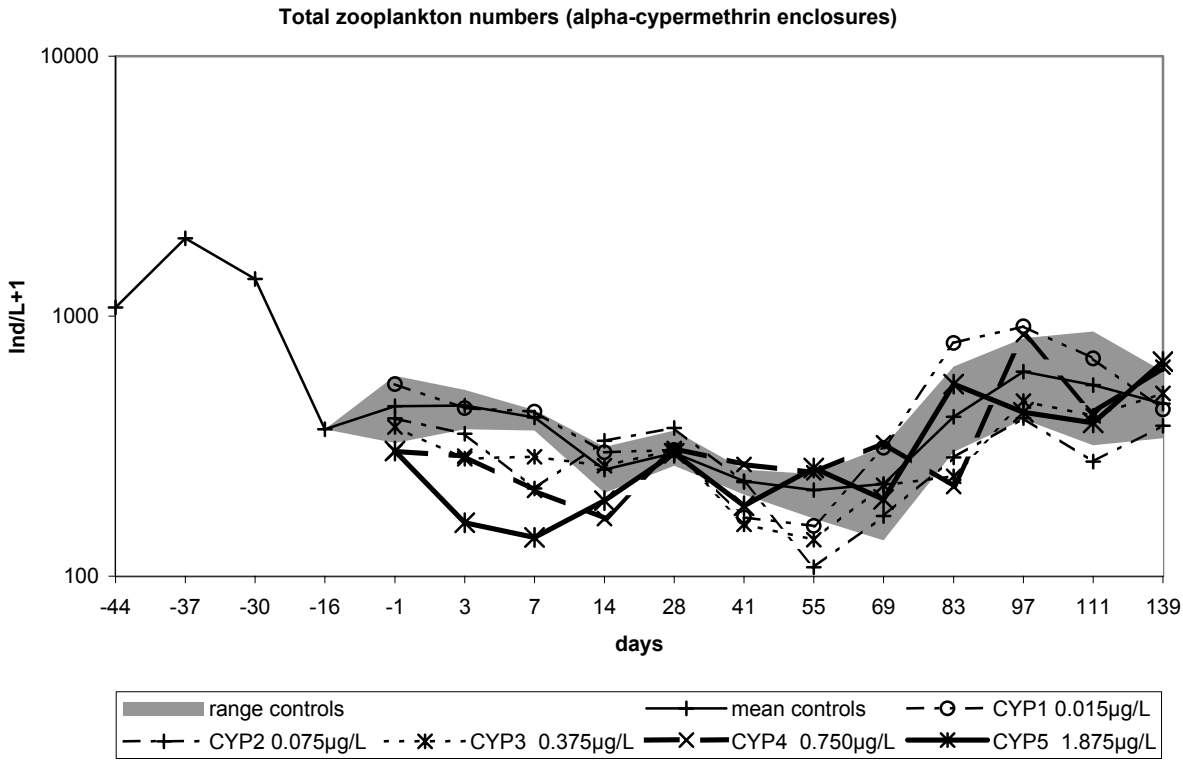
3.7.2 Abundance data

3.7.2a Total abundance

Figure 23 shows the total number of zooplankton organisms found in the pond system. Note that in the first year (top diagram) no major deviations are visible but for the first 14 days a.t., in which there was a decrease except in CYP1. In the second year, CYP5 has more zooplankton than the remaining enclosures except for the June sample. The other enclosures are below the control range most of the time.

NOEC is 0.015 µg/L, NEC lies between 0.141 and 0.204 µg/L, so it is less sensitive here. In the second year, a NOEC of 0.750 µg/L for an increase in the abundance with the treatment level, found by the Williams' test, can be stated (until June). However, all enclosures but CYP5 are more or less below the controls during that time. What can be seen here must be a secondary effect because the active ingredient is degraded in less than a month, even in CYP5. A reason may be the decreased numbers of *Ch. crystallinus* (Figure 25) that reach control level

in June 2001, too. This species is the top predator in the system and can execute top-down control on its prey. Less of this taxon can eventually lead to an increase in zooplankton. The fact that the treated enclosures except CYP5 have generally lower abundances than the controls in the second year may simply be due to a divergent development in these enclosures. It may not be addressed to the treatment. The same holds true for the increase in CYP5 on the last two sampling dates.



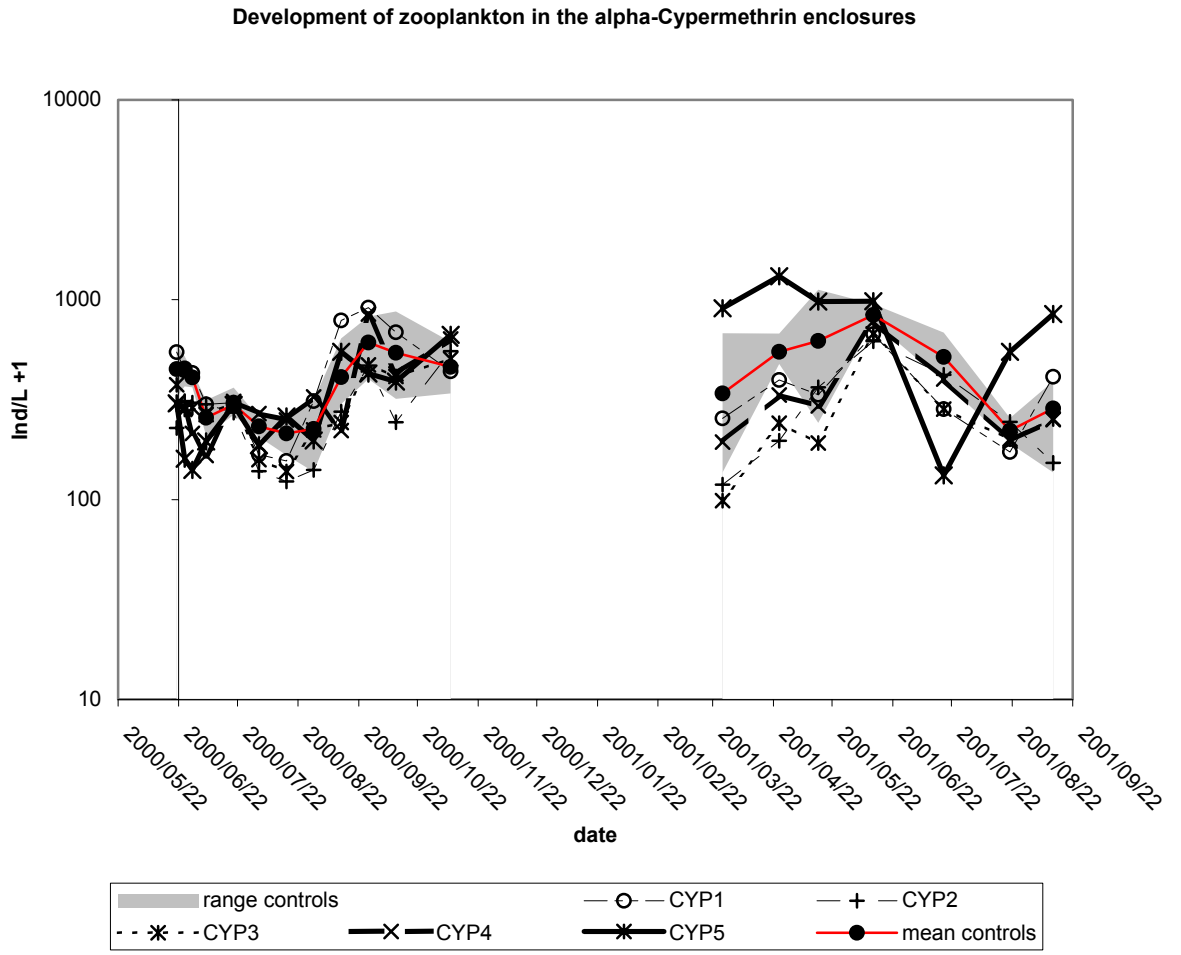


Figure 23: Total abundance of Zooplankton (CYP study); top: year one, bottom: both years

3.7.2b Species richness

The number of taxa found in zooplankton is about 16±3 (Figure 24). There are distinct differences between the enclosures right from the start of the study. An effect of the treatment cannot be noted.

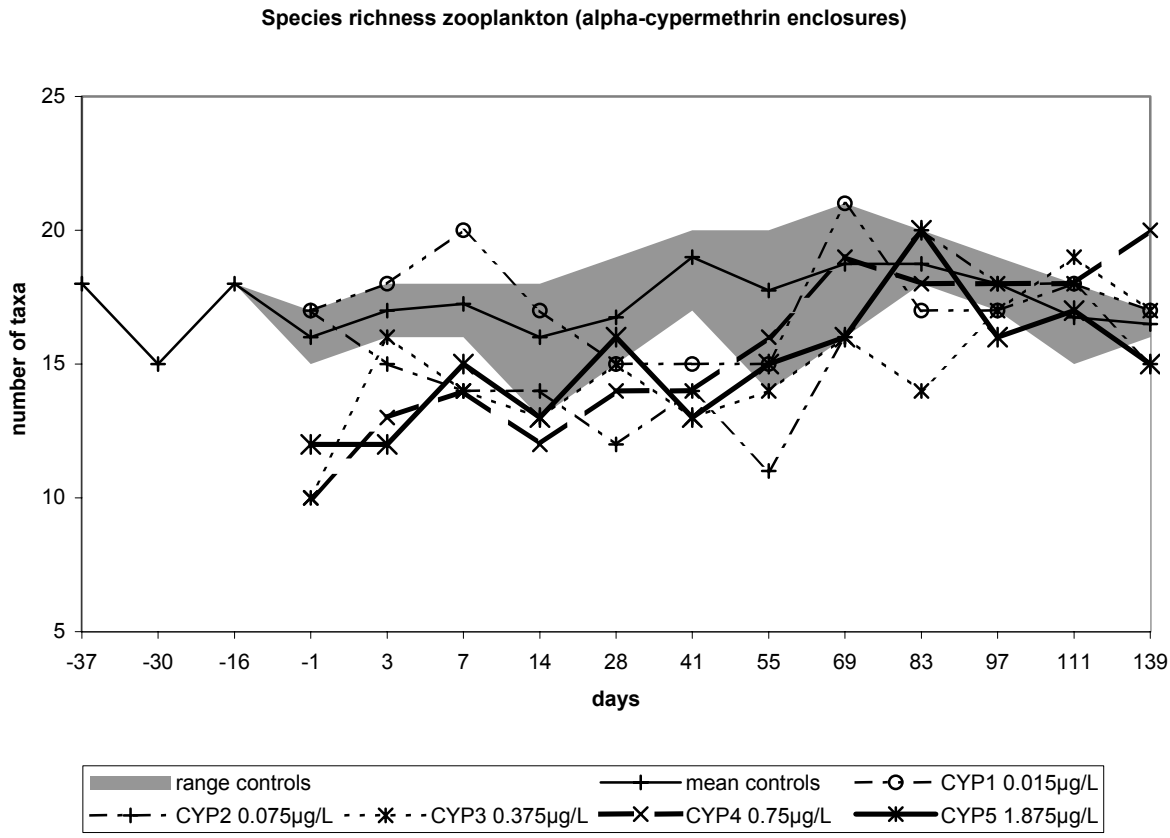


Figure 24: Number of taxa found in the zooplankton of the CYP enclosures

3.7.2c Chaoborus crystallinus

Since there are no fish in the test system, the top predator on zooplankton is the dipteran larvae *Ch. crystallinus* (LIAR 1990). It is very sensitive to the treatment (Figure 25).

Numbers are decreasing in all treated enclosures in the first 28 days a.t.. Up to day 69 a.t. CYP1 and CYP2 are in line with the controls again. From day 83 a.t. on (September 2000) no real changes in counts were found until April 2001. This is due to the life cycle of this species. No more eggs may hatch or are lied in autumn (MÜLLER 1995). In late spring (April to June, MÜLLER 1995), larvae hatch to adults which start reproducing right afterwards. Therefore, no effective change in numbers can be seen until June (CYP5 has an outlier in May 2002). This is when all enclosures reach control level again. In August 2001, the lower treated enclosures even over-compensate for the losses in the first year.

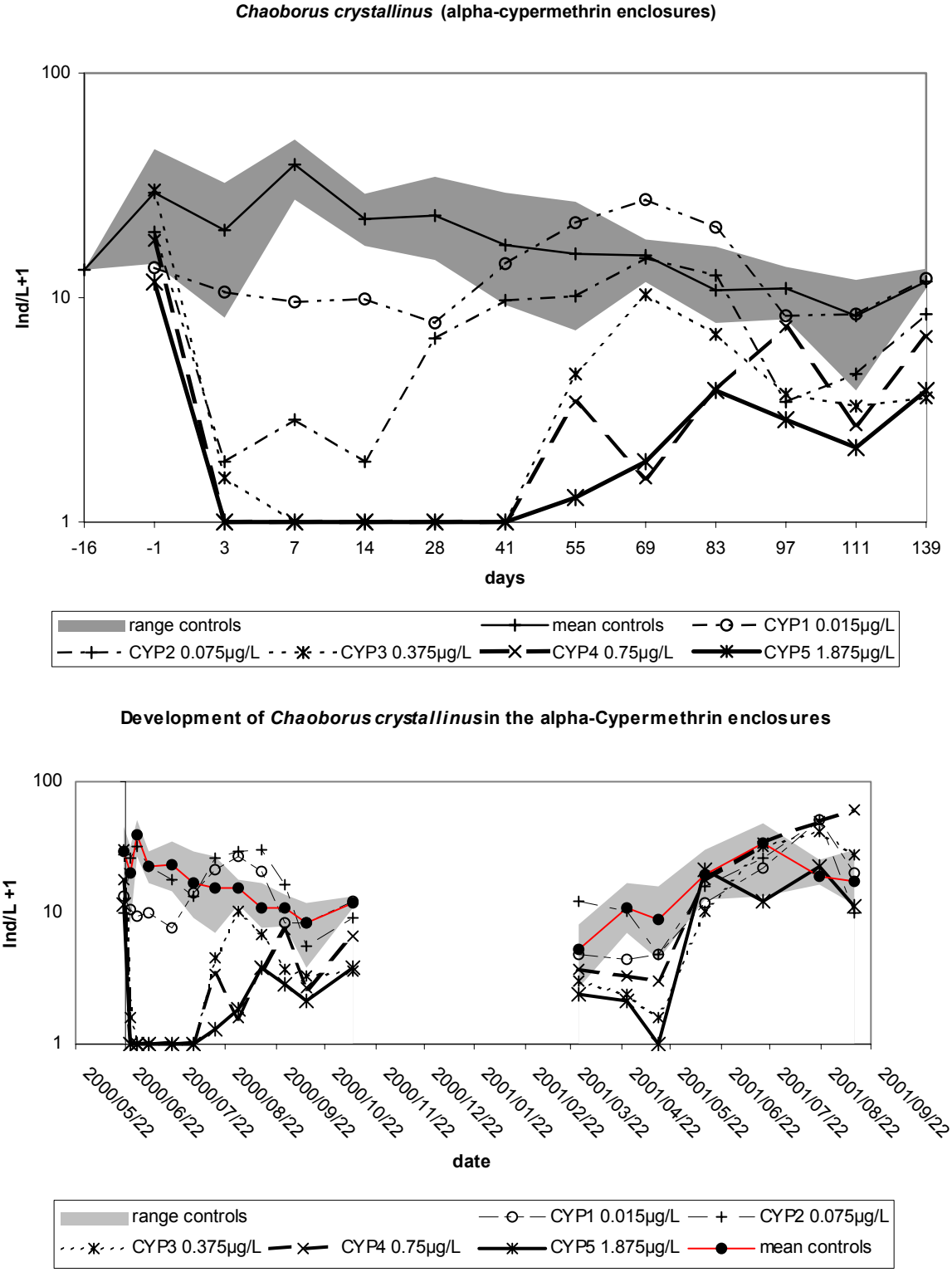


Figure 25: *Chaoborus crystallinus* in the CYP study; top: year one, bottom: both years

Thus, diminished predation pressure on zooplankton of CYP3 – CYP5 for about one year is the consequence.

NOEC for the species is lower than 0.015 µg/L and cannot be given here. NEC calculation shows values between 0.005, 0.009, and 0.035 µg/L corroborating the fact that abundance was already changed in CYP1.

3.7.2d Nauplii

Nauplius larvae of Copepods are an important taxon in these kinds of studies (Figure 26). Normally, Copepods have only 1 (-2) generations in a year (SOMMER 1994) so impacts on the larvae lead to prolonged treatment effects.

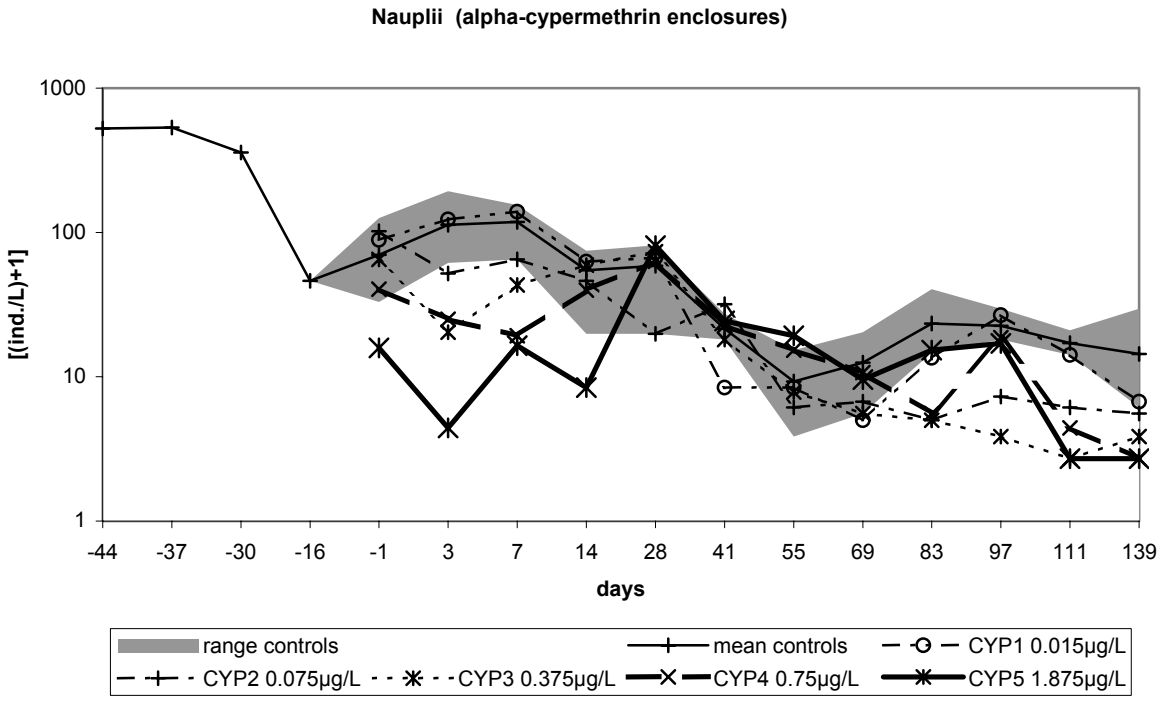


Figure 26: Development of Nauplius larvae in the CYP study

In the first week a.t. there are significant effects ($p<0.05$ in Williams' tests, decrease) beginning in CYP2. The NOEC is 0.015 µg/L a.i.. From day 28 a.t. to day 69 a.t. there are no more detectable influences. Later on (day 83 a.t.), numbers decrease again (same NOEC) until the end of the study season. In spring, no more differences are visible (also valid for the rest of the following year, data not shown).

It is remarkable that the first impact is treatment related but the second one is not any more. The greatest deviations for the latter effect can be observed in CYP2 and CYP3. Thus, these devaitaions can be interpreted as a direct and a secondary effect, respectively. The direct effect is the "normal" toxicity, whereas for the later differences a combination of influences is possible:

- reduced predation (3.7.2c) in the CYP4 and CYP5, therefore higher counts
- already more predation in CYP2 and CYP3
- recovery in CYP1, no more effects (NOEC!)

It must also be taken into account that treatment effects on the adults (Figure 27 and Figure 28) influence the supply of newborn larvae in the long run. The more abundant cyclopoids show almost no reaction to the treatment (3.7.2e), so a constant supply of new-born larvae can be anticipated.

3.7.2e Cyclopoida

For Copepods, let me start with the Cyclopoids (Figure 27). There is some fluctuation in their abundance, but no apparent effect except for CYP5. From day 55 to 83 a.t. a NOEC of 0.750 µg/L was calculated. More Cyclopoids are found in the highest concentration level. This, again, may be due to reduced predation from *Chaoborus*.

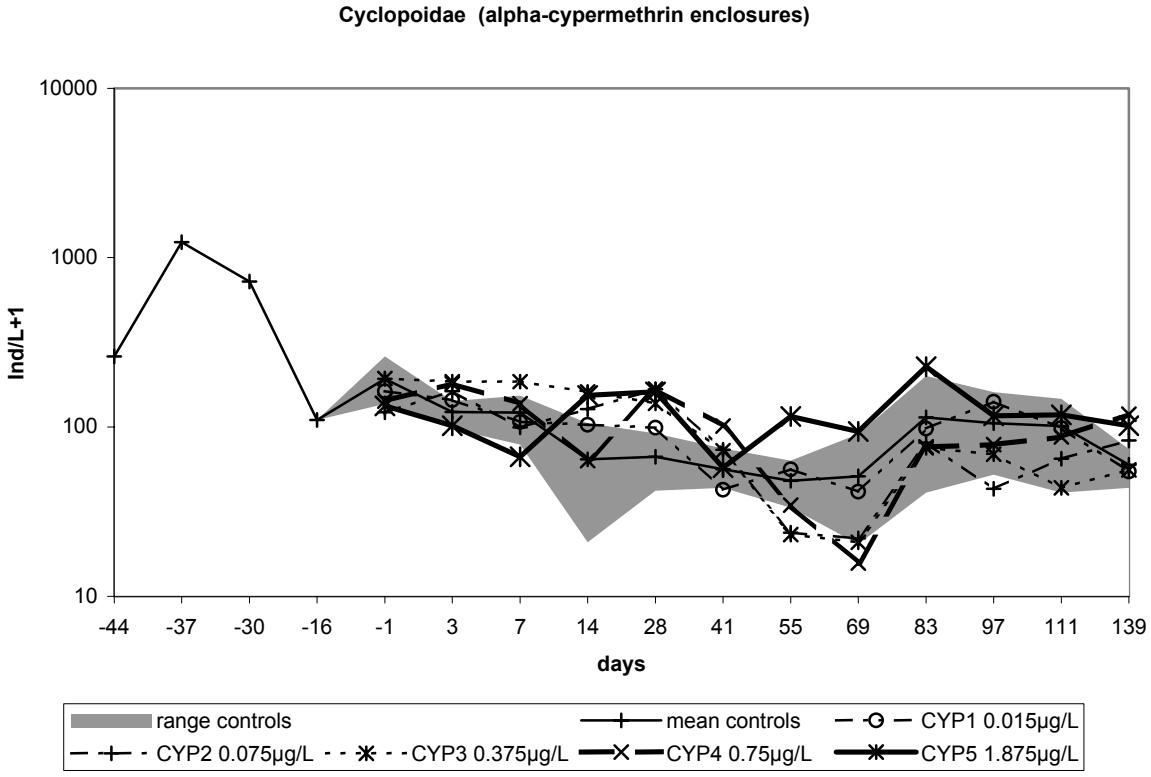


Figure 27: Cyclopoida (adults and Copepodits) in the CYP enclorures

There were no more effects in 2001. The numbers of this Copepod taxon are by far higher than of the following (*Eu. gracilis*). Influence on offspring of the Copepods is therefore bigger with Cyclopoida.

3.7.2f Eudiaptomus gracilis

With this calanoid a special reaction to the active ingredient is found (Figure 28). Numbers are very low at the time of the treatment (below 10/L), so the NOEC of 0.015 µg/L, though significant ($p<0.05$), must be dealt with care (MAISE 2002).

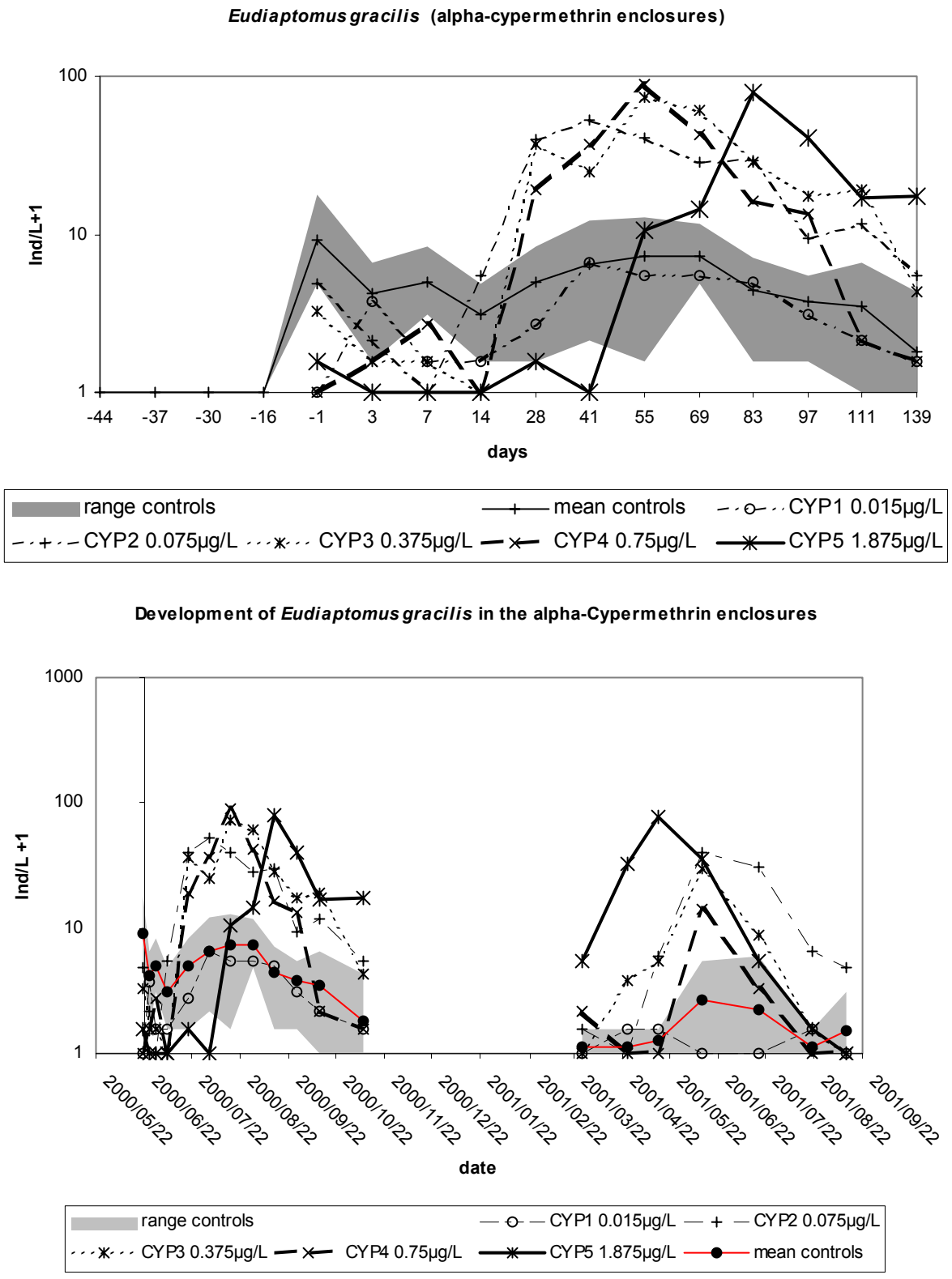


Figure 28: Numbers of *Eudiaptomus gracilis* (adults and Copepodits) in the CYP study; top: year one, bottom: both years

Abundance of *Eu. gracilis* in CYP1 stays within the control range during the whole study. The other enclosures show a big increase in the numbers of the calanoid. Gaining starts in CYP2 and CYP3 (day 28 a.t.), the higher treatment levels follow later in the year. This effect

more or less continues to the middle of the subsequent year. NOEC is 0.015 µg/L for this process.

This reaction can be explained in the following way: The species is sensitive to CYP (cf. 3.2) at concentrations used in the pond experiment. The active ingredient is degraded to a non-toxic level earlier in the lower concentration enclosures. Still, predation is lessened in all the highly treated ponds (Figure 25). Thus, numbers increase first in the lower concentration because in the others there are more CYP residues for a longer time restricting this augmentation. Only on proceeding decline of the a.i. can they go in line with the abundance in the lower treated enclosures. This effect lasts until the predator *Chaoborus* has acquired its "normal" abundance in the middle of the second year. *Eu. gracilis* abundances start getting lower from this point of time on. This is supported by a finding of PASTOROK 1980 (in LIAR 1990) that a Copepod is preferred to a Cladoceran by the dipteran larvae. Abundance of *Eu. gracilis* may thus be constricted by *Chaoborus* numbers.

In the second year, CYP4 reaches control range first and does not show increased numbers (but CYP2, CYP3, and CYP5 do). In the CYP4 enclosure, *Ch. crystallinus* numbers are a little bit higher than in CYP3 and CYP5 (Figure 25). So possibly, more predation limits *Eu. gracilis* there. CYP2 has more *Ch. crystallinus* and therefore less *Eudiaptomus* in autumn 2000 and spring 2001. Numbers in the calanoid increase again in 2001 when the predator gets less.

Numbers of *Eu. gracilis* are about one order of magnitude smaller than Cyclopoids. Thus, it is not probable that these effects can be seen in Nauplii (Figure 26).

3.7.2g Simocephalus vetulus

Big water flea are important grazers on phytoplankton (e.g. SOMMER 1994). In the test pond, *S. vetulus* plays this role since almost no daphnids were found. The reaction of the taxon towards treatment is given in Figure 29. It is sensitive to the toxin (treatment-related decrease between day 3 and 28 a.t.).

There is complete recovery in this taxon after one month (day 41 a.t.). This correlates with the generation time that is roughly one month, too (FLÖßNER 1972). Biomonitoring showed that no lethal toxic effects can be expected after just seven days (3.3.2).

NOEC is 0.075 µg/L. NEC is between 0.035, 0.062, and 0.244 µg/L. The curves are later on somewhat noisy but peaks cannot be addressed to any special treatment effect. The system is not fully silenced in this year, simply.

No great influence of *Ch. crystallinus* is expected for this species. At least the adults are too big to be a predominant prey (SWIFT 1992). The fact that it is the most abundant Cladoceran in the study is another prove for that (HEBERT 1982).

There are no effects to be seen any more in 2001.

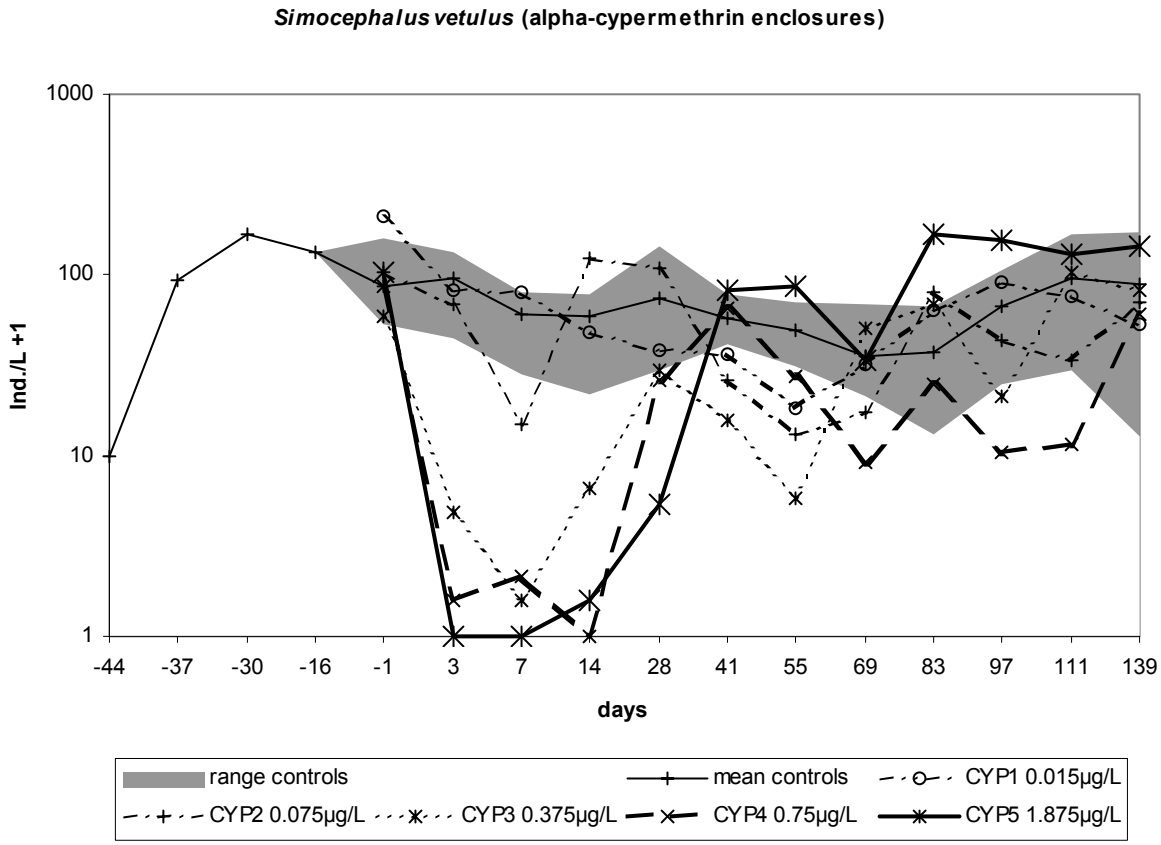


Figure 29: *Simocephalus vetulus* in the CYP enclosures

3.7.2h Chydorus sphaericus

Another Cladoceran, but this time a smaller one, is *Ch. sphaericus*. As shown in Figure 30, abundances were low in all enclosures in the first study year (except CYP1), but begin to rise in July. Hence, direct toxic effects cannot be explored[7].

Yet, what can be seen is a distinct increase over the control level in CYP5 beginning in October 2000 due to losses in the predator *Ch. crystallinus* (Figure 25). NOEC was calculated 0.750 µg/L, corroborating this finding. The higher abundance lasts until the middle of 2001 when predator numbers reach control level again (3.7.2c)[8].

[7] Note that in CYP5 up to August not one individual was found. CYP3 and CYP4 curves fall down right after application, too. So this species may well be sensitive!

[8] *Chydorus* is readily eaten by *Chaoborus* larvae instar III and IV (SWIFT 1992).

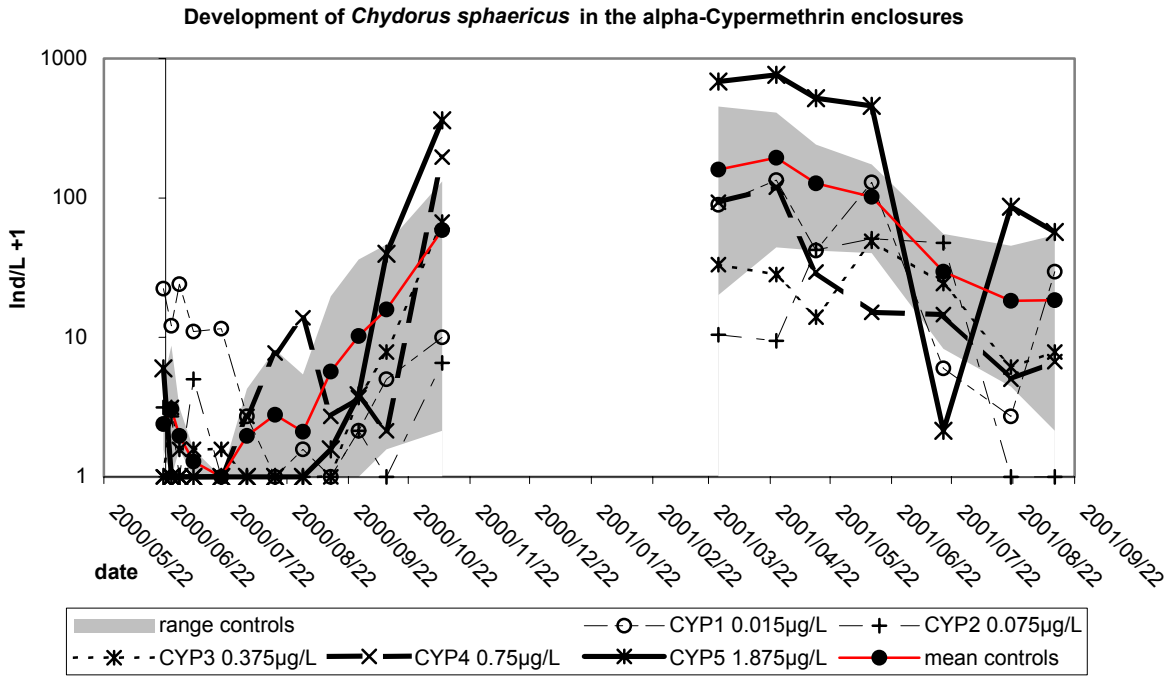


Figure 30: Development of *Chydorus sphaericus* (CYP)

3.7.2i Rotifera

Rotifera are very small grazers on picoplankton and bacteria. They have a short generation time about 1 week (STREBLE and KRAUTER 1988, KIRK 1997). CYP effects are shown in Figure 31.

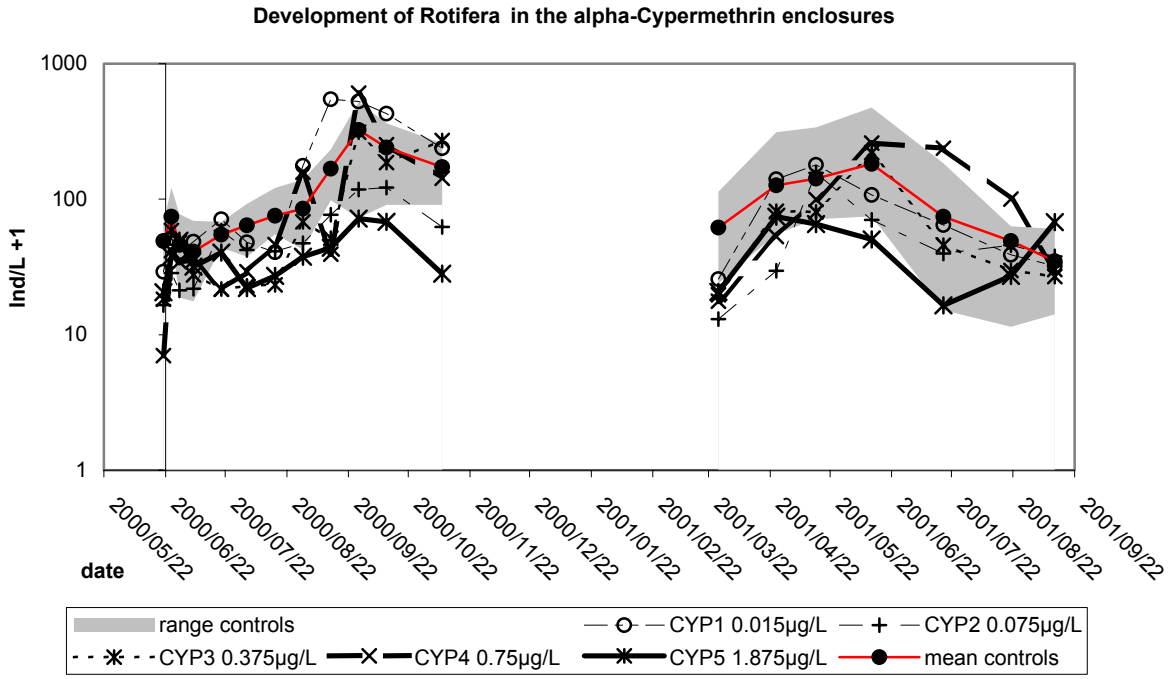


Figure 31: Progression of the Rotifers under CYP treatment

There is not too much of a reaction to the treatment seen here. Differences rather stem from enclosure deviations than from treatment. The NOEC of 0.750 µg/L (the same as *Ch. sphaericus*) is indicating this fact.

Since Rotifera are one of *Chaoborus'* main nutrition (LIAR 1990), somewhat surprisingly its reduced abundance (Figure 25) shows no effect[9] here. They seem to be completely under competitive control of the other, bigger grazers[10] (e.g. *Ch. sphaericus, Eu. gracilis*; see e.g. VANNI 1986). Even reduced numbers in CYP5 underline this interpretation.

3.7.3 Community analysis

3.7.3a Shannon index and evenness

Shannon index and evenness yield more or less the same curves, so the display is restricted to evenness (Figure 32, rationale see above). The theoretical H_{max} of the Shannon index was 3.04. Mean H_S in the treated enclosures is 1.64 and 1.89 in the controls; mean values for the evenness are 0.60 for the treated enclosures and 0.66 for the controls.

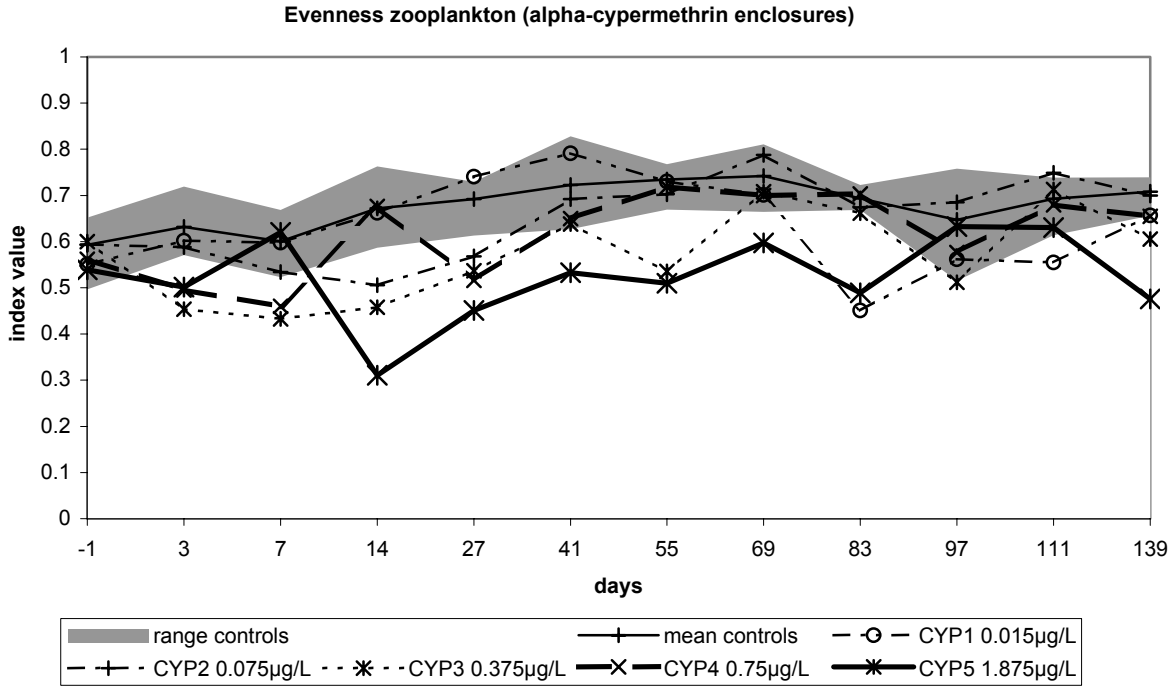


Figure 32: Evenness of zooplankton in the CYP study

Please note that differences between the enclosures are starting on day 3 a.t.. The evenness is reduced more or less treatment-related until day 97 a.t. As far as the treatment correlation is concerned, CYP4 does not go along with a steady trend with CYP the amounts. However, such a trend can be seen in some of the presented taxa (3.7.2). From 97 a.t. on, the endpoint is in line with the controls again. The pattern of the curves on days 111 and 139 a.t. does not give reason to assume a treatment effect. NECs are given in Table 21.

[9] It could be expected that they would increase because the numbers of the predator are lowered by the treatment.

[10] Already at their control abundance!

Table 21: NEC of Shannon index and evenness of the zooplankton in the CYP study

NEC [µg/L CYP]		
Evenness	**Shannon**	**N**
0.004	0.004	5
0.019	0.008	5
0.130	0.035	5

In short, these indices are clearly exhibiting an effect. With NEC values that are similar to the concentrations of CYP1 (0.015 µg/L) or below them, these analyses are among the most sensitive ones.

3.7.3b RAD index

The RAD index may reach a maximum of "2" (totally different samples). Here in Figure 33 the highest point is 1.24 (day 14 in CYP5). A distinct treatment effect can be stated for all enclosures but CYP1 until day 41 a.t.. Then CYP5 is not the most affected treatment level anymore but the ones "in the middle". This finding may result from secondary effects: Less predation (3.7.2c) and already no more toxic effects of any kind. From day 97 to day 278 a.t. CYP5 is the only pond not in the control range. This fact can be due to increased numbers of smaller caldocera and *Eu. gracilis* together with the decrease in Rotifera and *Ch. crystallinus*. The later deviations should not be addressed to the treatment; this may be over-interpreted[11] much too easily. However, such late consequences have been seen in the RAD analysis of the phytoplankton (3.6.3b).

It is concluded that, by one year after the application, this parameter shows recovery to the control range. It cannot be excluded, though, that additional consequences of the treatment alter the zooplankton community late in the second year.

[11] Although there might be a link to *Chaoborus* again. The predator reaching "normal" abundances again may lead to changes in a biocoenosis adapted to lower predation pressure (i.e. a secondary effect of the treatment late in the second (!) year.

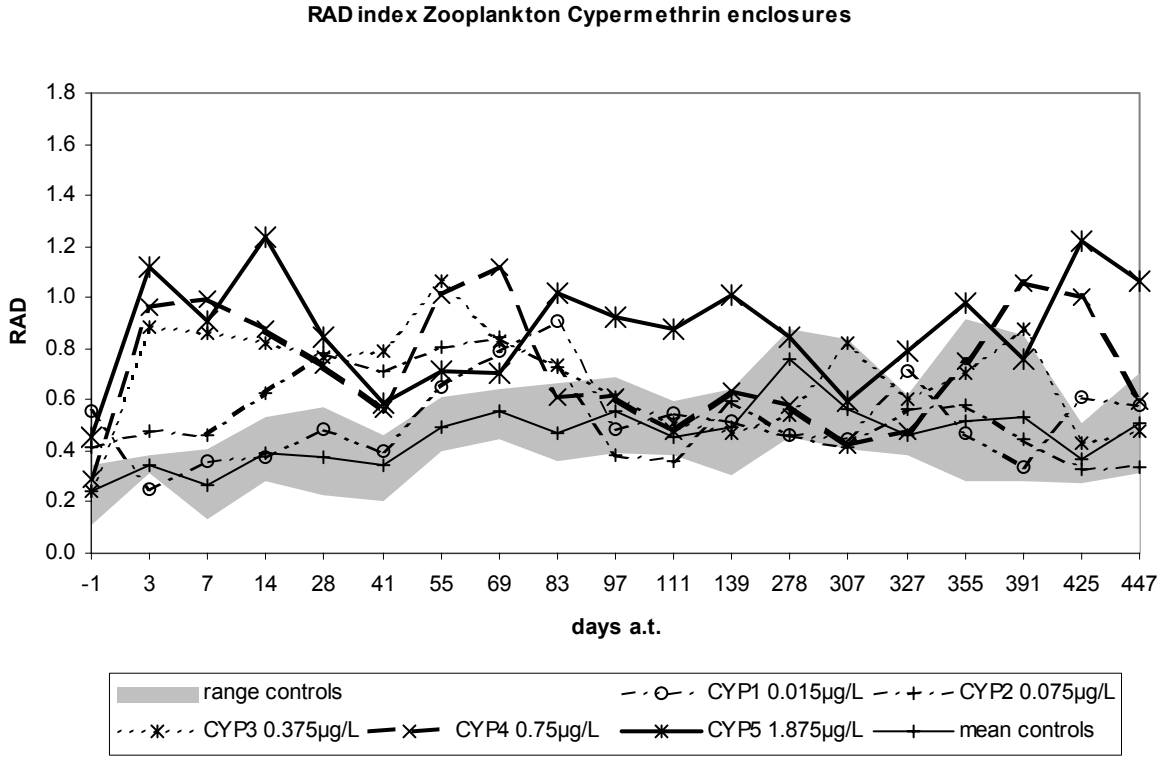


Figure 33: RAD index zooplankton (CYP)

3.7.3c PRC analysis

PRC curves for zooplankton are shown in Figure 34. Again, there are some differences between the enclosures even on day –1. What can clearly be seen, though, is that all treatment levels but CYP1 (0.015 µg/L) show an impact of CYP. The cdt values are increasing, but not always in a treatment related way. By day 97 a.t. the system has recovered to the initial differences except for level CYP5. Care has to be taken with that finding because in autumn[12] abundance in the controls tends to go down as well. This may lead to a misinterpretation of a recovery. When looking at the total abundances (Figure 23), this can be excluded. There is a real recovery except for CYP5.

This analysis is significant: p=0.005. It explains 38.7% of the variances by the treatment of which 25.9% are displayed. The $NOEC_{community}$ is smaller than 0.015 µg/L, being more sensitive than what would be guessed from looking at the curves alone. NEC is at (0.006)-0.021-(0.029) µg/L (lower-mean-upper value). These results fit in with the ones from *Ch. crystallinus* (3.7.2c) and the total abundance (3.7.2a) proving the power of the analysis.

Species found important for the system reaction to CYP are listed in Table 22.

[12] Day 97 is late September!

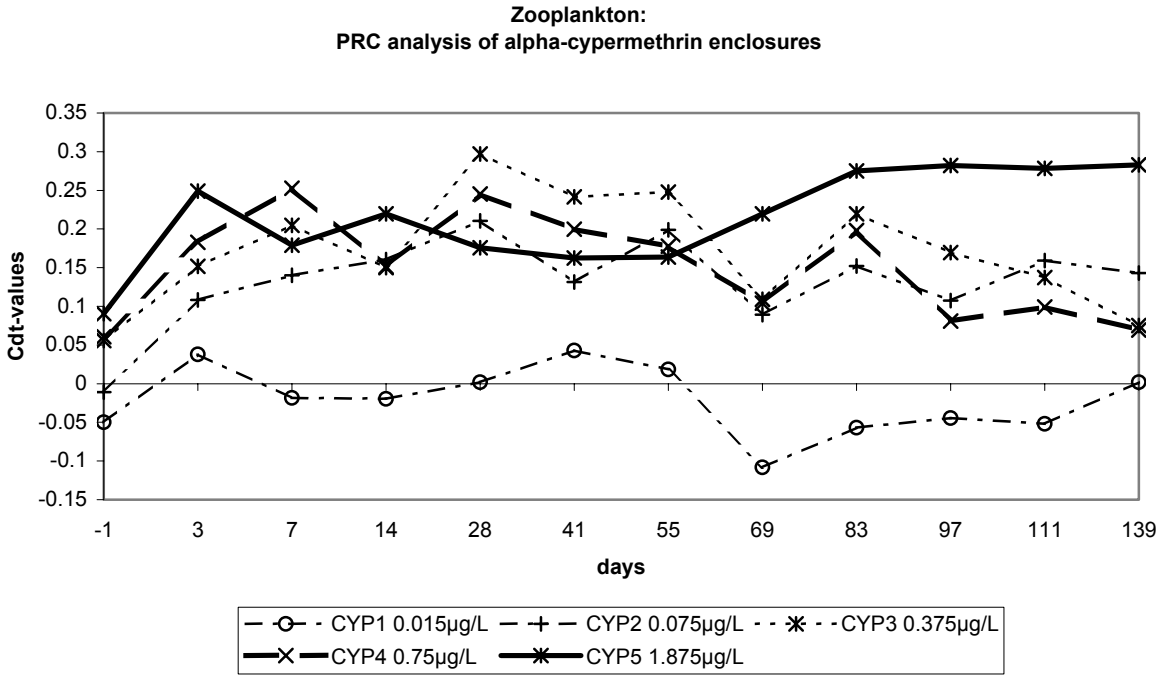


Figure 34: PRC curves of zooplankton in the CYP enclosures

Table 22: Zooplankton taxa with species score >0.5 (absolute value) in the CYP study

Taxon	species score
Chaoborus crystallinus (Insecta)	-0.99
Mytilina mucronata (Rotifera)	-0.73
Lepadella ovalis s.l. (Rotifera)	-0.64
Simocephalus vetulus (Cladocera)	-0.51
Eudiaptomus gracilis (Copepoda)	0.62

Here we find the ones showing clear effects for themselves (*Ch crystallinus, S. vetulus, Eu. gracilis*) and two Rotifers. They were found in rather low numbers and display no clear effects via "classical" analysis. It can be stated that they, in a way, follow the pattern of the total number of Rotifera.

3.7.4 Overview of treatment effects of CYP on the zooplankton

A summary of NOECs of important endpoints is presented in Table 23.

Table 23: Summary of NOECs of zooplankton (CYP study)

Taxon	NOEC [µg/L CYP]	remark
Chaoborus crystallinus (Insecta)	<0.015	very sensitive
Chydorus sphaericus (Cladocera)	0.75	secondarily increased
Cladocera	0.075	sensitive
Cyclopoida (Copepoda)	0.75	almost no effect
Eudiaptomus gracilis (Copepoda)	0.015	special pattern, secondary effects
Nauplia ssp. (Copepoda)	0.015	sensitive
Rotifera	0.750	almost no effect
Simocephalus vetulus (Cladocera)	0.075	sensitive, main Cladoceran
total abundance	0.015	direct and secondary effects
$NOEC_{community}$	<0.015	no recovery in the first year

There are some taxa that show sensitivity towards CYP. Of special importance are the reactions of *Ch. crystallinus* (Insecta) and *S. vetulus* (Cladocera). The first one is the top predator (LIAR 1990) in the system (NOEC<0.015 µg/L), the latter one presumably one of the most efficient grazers (NOEC=0.075 µg/L) because phytoplankton increases when it is reduced (3.6.2a). CYP treatment reduces the numbers of both zooplankton taxa. The Cladoceran recovers within an month's time in all enclosures; the dipteran larvae requires about one year for that.

These findings are in line with the generation time of the animals. As mentioned above (3.1), direct toxicity is limited to about 2 weeks a.t.. Generation time of the caldoceran is about 2-3 weeks (FLÖßNER 1972). Together with the biomonitoring results (3.3.2) a quick recovery can be expected. So here this is a "class 3" effect (BROCK *et al.* 2000 in EU 2002): short-termed with recovery within 8 weeks.

Ch. crystallinus is very susceptible towards CYP (see 3.2, 3.3.1, and 3.7.2c). Its development with time can be explain in this way: MÜLLER (1995) mentions up to two generations per year for this species, the first in April – June and the second between August and October. Adult females lay eggs almost immediately after hatching and copulation. RATTE (1979, 1985 in BÜNS and RATTE 1991) found the optimum temperatures for the development of the larvae at between 14°C and 25°C. Looking at the water temperatures in the enclosures (Figure 7, page 42), this optimum is not met between September and May.

Hence the second generation, which should fill up the lack of larvae caused by the treatment, is

a. reduced by the treatment when they are still small larvae and will not reach "normal" abundance as adults (means less "internal" supply),
b. delayed in development by the temperature (leading to less supply from uncontaminated ponds).

As a result treatment effects cannot be compensated before the first generation of new larvae starts growing in May-June in the following year. This is exactly the pattern found here in the three high treatment levels. A class 5 - pronounced long-term - effect according to BROCK *et al.* (2000 in EU 2002) is found in CYP3 to CYP5. Abundances of *Ch. crystallinus* show class 3 effects in CYP1 and 2.

Secondary effects are triggered by the development of *Ch. crystallinus* to the treatment: Predation pressure is lessened. This allows some taxa to gain in abundance until the quantity of the top predator *Ch. crystallinus* is in line with the controls again. In *Eu. gracilis*, this reaction is modified by direct toxicity correlated to the residual amount of CYP. Planctonic algae also relate to the fluctuations in *S. vetulus* (see above).

Species richness in zooplankton is not affected by the treatment. Surprisingly, evenness and Shannon index are very sensitive. Their NECs are approximately the CYP1 level (0.015 µg/L).

RAD analysis finds recovery one year a.t. for CYP5. The other treatment levels reach the control range at day 97 a.t.. PRC backs these findings. The $NOEC_{community}$ is smaller than 0.015 µg/L proving the applicability of this method by finding the most sensitive species, *Ch. crystallinus*.

3.8 Summary of the CYP study

There are clear effects of the treatment, direct as well as secondary ones. Most important for the impact on the system is

a. the very high sensitivity of the top predator *Chaoborus crystallinus*,
b. the quite sensitive reaction of the grazer *Simocephalus vetulus,*
c. the secondary increase in the numbers of other grazers, especially *Eu. gracilis* later in the study.

Biomonitoring data (LC_{50}) and field toxicity match quite well. Laboratory LC_{50} for the Cladoceran indicate higher resistance than found in the outdoor experiment. Data for the dipteran larvae correspond better. Differences in field toxicity between *S. vetulus* und *Eu. gracilis* are corroborated by their single species tests. *Eu. gracilis* is less sensitive towards CYP in both tests. This detail may facilitate the increase in *Eu. gracilis* making it the better competitor under pesticide influence (sublethal effects: reduced grazing ability, see DAY and KAUSHIK 1987).

There were no effects on water quality parameters.

Hence, all visible (secondary) treatment effects can be explained by less predation on grazers thus altering phytoplankton. The system reaction shows a clear top-down control. An over-all NOEC can be set up to 0.015 µg/L a.i. or even lower (based on the most sensitive parameters *Ch. crystallinus*, evenness/Shannon index, and PRC).

Figure 35 depicts the sector of the food web that was altered by CYP treatment. The grey triangle indicates decreasing sensitivity towards CYP, the arrows the interaction of the components.

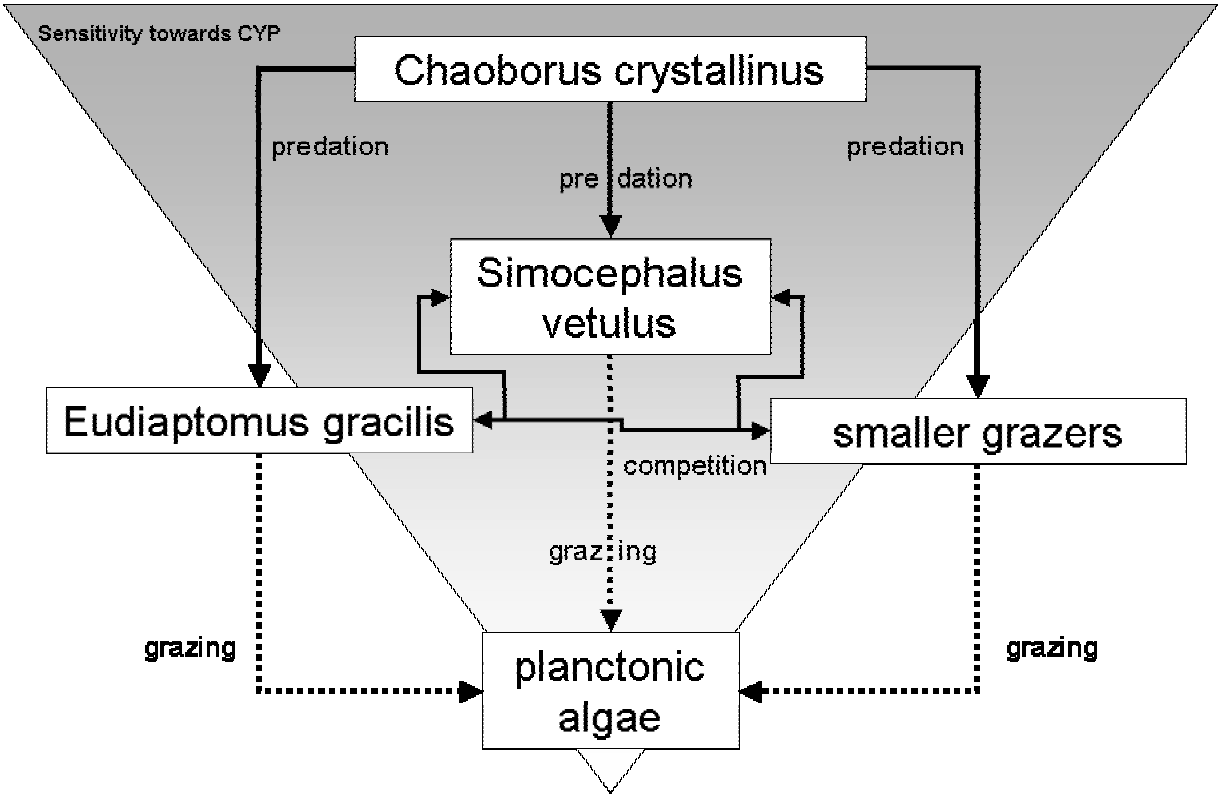


Figure 35: Model sector of the food web interaction under CYP influence (thick lines: predation, thin lines: competition, dotted lines: grazing; triangle: sensitivity towards CYP)

When the top predator is reduced in numbers at a certain amount of CYP, less susceptible grazers will increase, being not negatively influenced by the treatment. As a consequence, numbers of algae will go down. If the amount of CYP is high enough to cause a decline in grazers as well, phytoplankton may increase. These reactions are somewhat altered by competition between grazers and their ability to feed on certain algae, generative behavior of the taxa, and slight direct impacts of CYP on certain algae.

Secondary effects were still visible to some extend in the second year, long after the a.i. itself had disappeared from the water column.

4 Results and discussion of the Isoproturon study

4.1 Herbicide residues

About 95.4% (200 mL sample) and 95.0% (100 mL sample) of the herbicide could be retrieved by the measurements. The limit of detection (LOD) was about 12 µg/L when injected directly. Together with the SPE enrichment this gives a resulting limit of about 50 ng/L. Values had a standard deviation of about 2%. Figure 36 shows the decline of IPU together with some of the results of the regression analysis (Table 24).

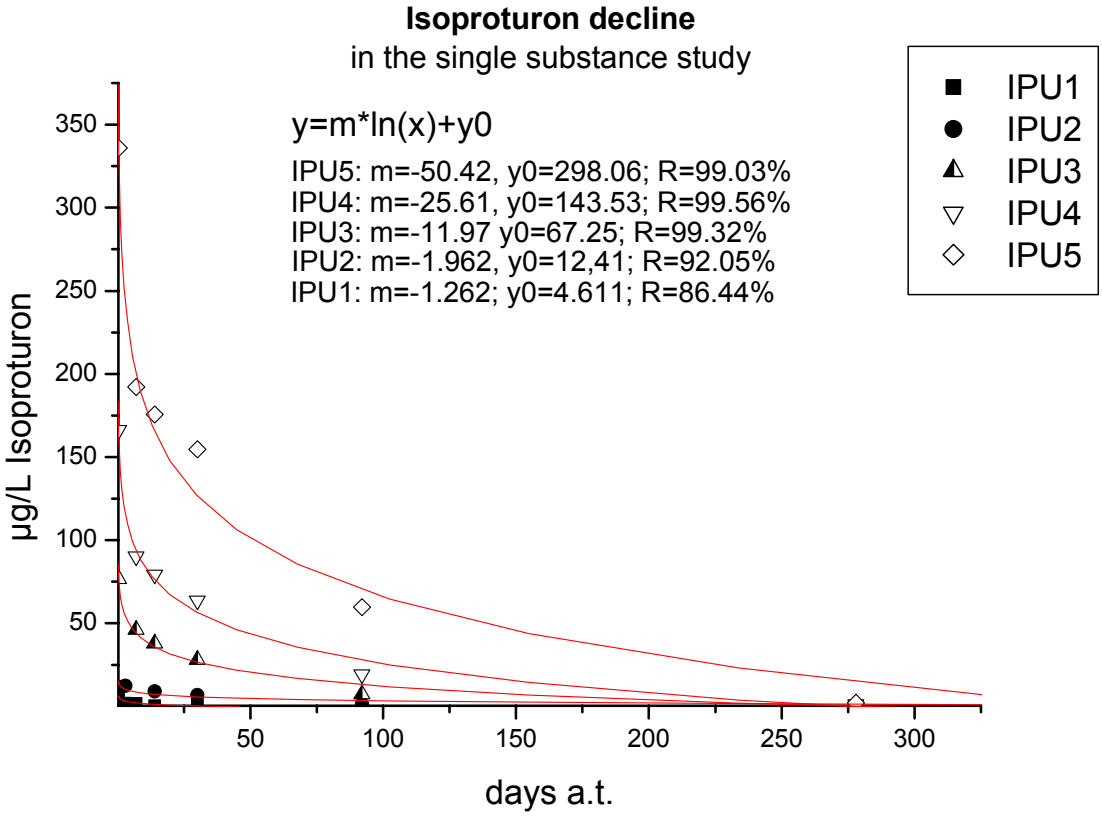


Figure 36: IPU residues (single substance study) and regression data

In this figure and in Table 24, y0 are the calculated values for IPU on time=0. The planned levels (4, 16, 64, 128, and 256 µg/L) were fully met. The quality of the regression (R-value) is quite good, too (mostly above 90%). The component in IPU5 has completely vanished from the water column after about one year. The time until no more herbicide is supposed to be in the enclosure water is realistic except for IPU2. DT_{50} value is approximately 16 days (Table 24).

Table 24: DT_{50} values and concentrations regression data of IPU, model $y = m * \ln(x) + y0$

	IPU1	IPU2	IPU3	IPU4	IPU5	mean±standard dev.
y0 [µg/L]	4.61	12.41	67.25	143.53	298.06	
y0 planned [µg/L]	4	16	64	128	256	
% of planned	115.3	77.6	105.1	112.1	116.4	105.3±16.1
m	-1.262	-1.962	-11.97	-25.61	-50.42	
R [%]	86.44	92.05	99.32	99.56	99.03	95.3±5.9
SD	1.41	2.21	3.58	6.11	17.99	
p	0.059	0.009	<0.0001	<0.0001	<0.0001	
DT_{50}	6.22	23.62	16.59	16.48	19.22	16.43±2.86
time for detoxification [days]	39	558	275	272	369	

All these results are in line with the literature; e.g. MERLIN, VUILLOD *et al.* 2002 found a half life of between 15-35 days and ESER 2001 10-20 days. IPU is therefore rather persistent in the test system.

4.2 Single species tests

No effects of IPU were seen on *Eudiaptomus gracilis* and *Simocephalus vetulus* up to 1000 µg/L a.i. after 48 hours. The herbicide is not toxic for these crustaceans. These findings are supported by the results of TRAUNSBURGER *et al.* 1996. They found *Daphnia magna* to be insensitive up to 1000 µg/L IPU.

4.3 Biomonitoring

Monitoring of survival of the species *Chaoborus crystallinus, Simocephalus vetulus,* and *Daphnia pulex* showed no effect of IPU. The herbicide exerts no negative impact on the zooplankton species investigated. Example data is presented in Table 25. These finding back the results of the single species tests (4.2). The experiment with *Chaoborus crystallinus* can only be evaluated to a minor extend, because too many of the animals in the controls died. However, no dead animals were found in the IPU treated water in this experiment. It can therefore be concluded that IPU is not toxic for the insect larvae.

Table 25: Biomonitoring with IPU, water taken 6 h a.t., 24 h examination

amount IPU [µg/L]	Chaoborus % dead	Simocephalus % dead	Daphnia % dead
0	80	0	0
0	60	0	0
0	0	0	0
0	0	0	0
4	0	0	30
16	0	0	10
64	0	20	9
128	0	0	0
256	0	0	0

4.4 Water quality parameters

4.4.1 Alkalinity

Alkalinity is a parameter describing the capacity to buffer changes in the pH (SCHWOERBEL 1999). Here, in Figure 37, it is expressed as the amount of bound CO_2. It is linked to the photosynthesis taking place in the pond system. More primary production results in less bound CO_2 in the water and vice versa (SCHWOERBEL 1999). Replenishing of the amount of bound CO_2 can be assumed constant over time, so alkalinity can be seen as an indicator for the productivity in the test system. An increase in alkalinity is indicating a decrease in primary production.

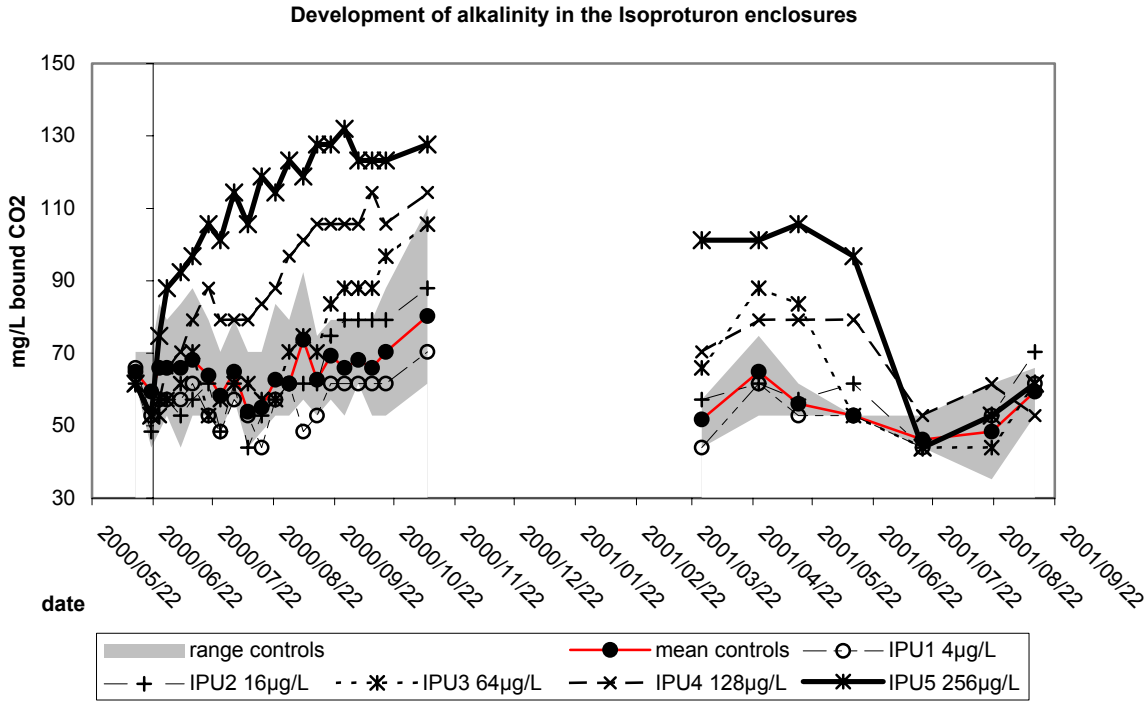


Figure 37: Alkalinity in the IPU study

Alkalinity starts increasing in IPU5 already in the first week a.t.. IPU4 follows in July (one month later) and IPU3 in September (3 months later). The impact lasts up to July 2001. It is therefore a class 5 effect (BROCK *et al.* 2000 in EU 2002). Williams' test found a NOEC of 4 µg/L IPU. The statistical evaluation is more sensitive than looking at the curves alone (IPU2 may not be regarded as different from the controls). NEC is somewhat higher, ranging between 37.6 µg/L and 84.0 µg/L (20 samples).

Recovery corresponds with detoxification (Table 24). IPU3 is in line with the controls a little bit earlier (in June). The time span for the parameter getting back to "normal" also relates to the temperature (Figure 7, page 42). It takes some time for algae and especially the macrophytes to grow (4.5 and 4.6), in particular when temperatures are rather low. So photosynthesis in the system reaches control values later than on the actual time when no more herbicide is present in the water.

4.4.2 Conductivity

This parameter tells us about the number of ions dissolved in the enclosure water. In Figure 38 shows almost the same picture as the alkalinity. IPU5 starts having significantly increased values ($p<0.05$ in Williams' test) from day 7 onwards. The maximum of 292 µS/cm is reached on day 139 (last sampling in 2000). Recovery is seen on day 425 a.t., in August 2001. The lower treated enclosures follow this pattern showing a later beginning and an earlier recovery. This is corresponding to the detoxification data again (Table 24). IPU1 shows no effects. NOEC is 4 µg/L IPU, the lowest NEC 42 µg/L[13].

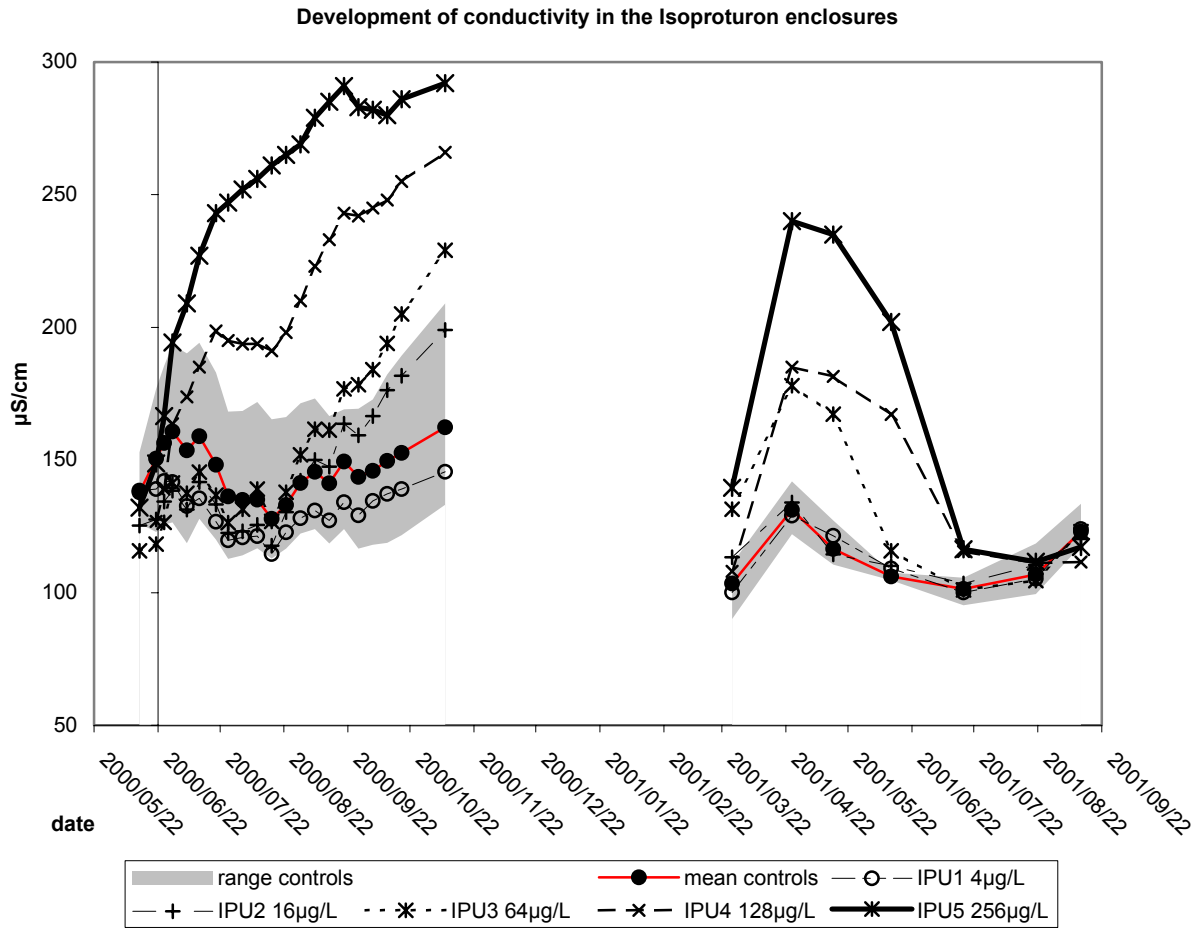


Figure 38: Conductivity in the IPU study

Explanation can be given by reduced primary production under herbicide influence (see also 4.4.3 oxygen content). By this process, more ions remain dissolved in the water. Additionally, degrading of the macrophytes (and algae) in IPU3 and higher (see 4.5) contributes to the increase.

The parameter recovered over one year after treatment. Thus, IPU imposed a severe impact on it (class 5 effect according to BROCK *et al.* 2000 in EU 2002)

[13] The other values are app. 63 and 101 µg/L, but IPU3 and IPU4 do show effects. As a result, these values are neglected.

4.4.3 Oxygen content

Photosynthesis is an important process providing the water in the test system with oxygen. The functional parameter evaluated here gives a good idea of how much primary production is going on in the system. So a reduction under herbicide influence is expected and indeed seen in many comparable studies (e.g. HUBER *et al.* 1995, KERSTING and VAN DEN BRINK 1997, EBKE 1999, ESER 2001).

The amount of oxygen in the water decreases with IPU treatment (Figure 39). Oxygen saturation exhibits the same picture and is not shown separately. The lowest measurement is on day 20 a.t. in IPU5 with 1.8 mg/L (19.5 % saturation). The actual amount of oxygen is linked to the abundance of algae in the enclosures (see 4.6.2a). Whenever there are more algae in the enclosure, the oxygen content goes up - at least to some extend. However, the total abundance of planktonic algae does not shows such a clear dose-response pattern like the oxygen content. This can be interpreted in the following way:

On the one hand, algae die because of the treatment. On the other hand, those who survive the treatment are merely able to do photosynthesis at a reduced level. As a result, the oxygen content is much more treatment related than the algal abundance alone.

Twelve times the NOEC was calculated 4 µg/L. NEC corroborates this result. Values are 6.86 µg/L, 7.39 µg/L, and 12.89 µg/L. Regressions were possible on 12 sampling dates during both years. All in all oxygen is a very sensitive parameter. Treatment exhibits impact on the system for more than 8 weeks. So it has to be classified a class 5 effect according to BROCK *et al.* 2000 (in EU 2002).

What is even more interesting is the increase in oxygen beginning in June 2001. The three highly treated enclosures show this reaction with the biggest increase in IPU4 (NOEC 16 µg/L for the process). This, again, is in line with the degrading of the herbicide (Table 24). It may be explained by extensive growth (and photosynthesis) of macrophytes (4.5), since no real increase in algae was found (4.6.2a). Macrophytes were reduced by the treatment and therefore have good growing conditions, e.g. in space, light, and nutrition.

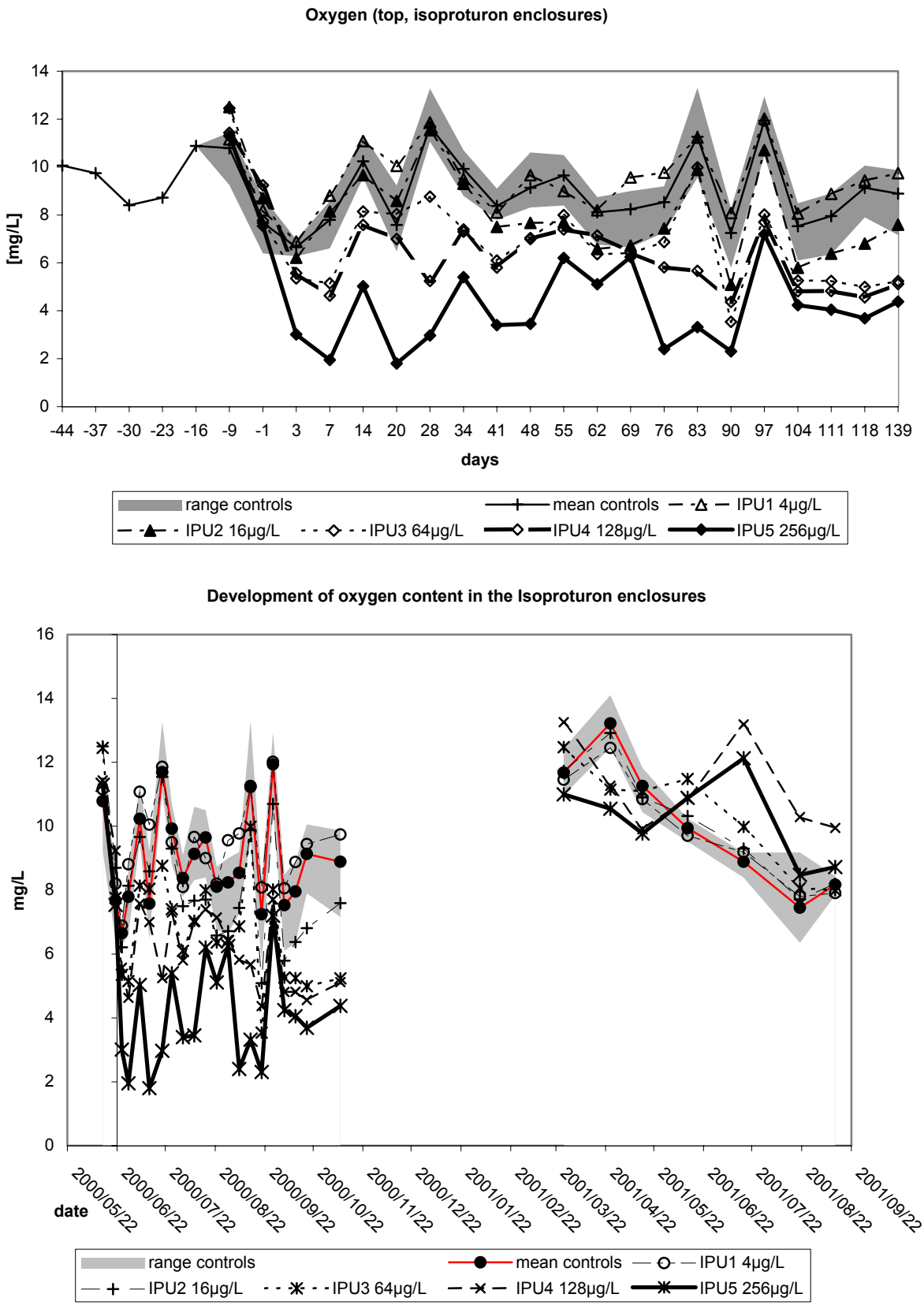


Figure 39: Oxygen content in the IPU study; top: year one, bottom: both years

4.4.4 pH value

The pattern in pH is analogous to the one in oxygen (Figure 40). Controls show a value of about 8.5-9 over the whole study. Treated enclosures, beginning in IPU3, have a more neutral pH. NOEC is 4 µg/L (7 occasions). NEC minimum values are in this range, too: 1.61 µg/L, 4.20 µg/L, and 9.65 µg/L IPU. In 2001 an increase above the control level can be seen in IPU4-5, beginning in July (data not shown, alike oxygen). This effect lasts until the end of the study. Maximum values are slightly below pH 10. As stated above (4.4.3 Oxygen content), this is related to the photosynthesis in the ponds. More primary production eventually leads to a high pH in the water (SCHWOERBEL 1999).

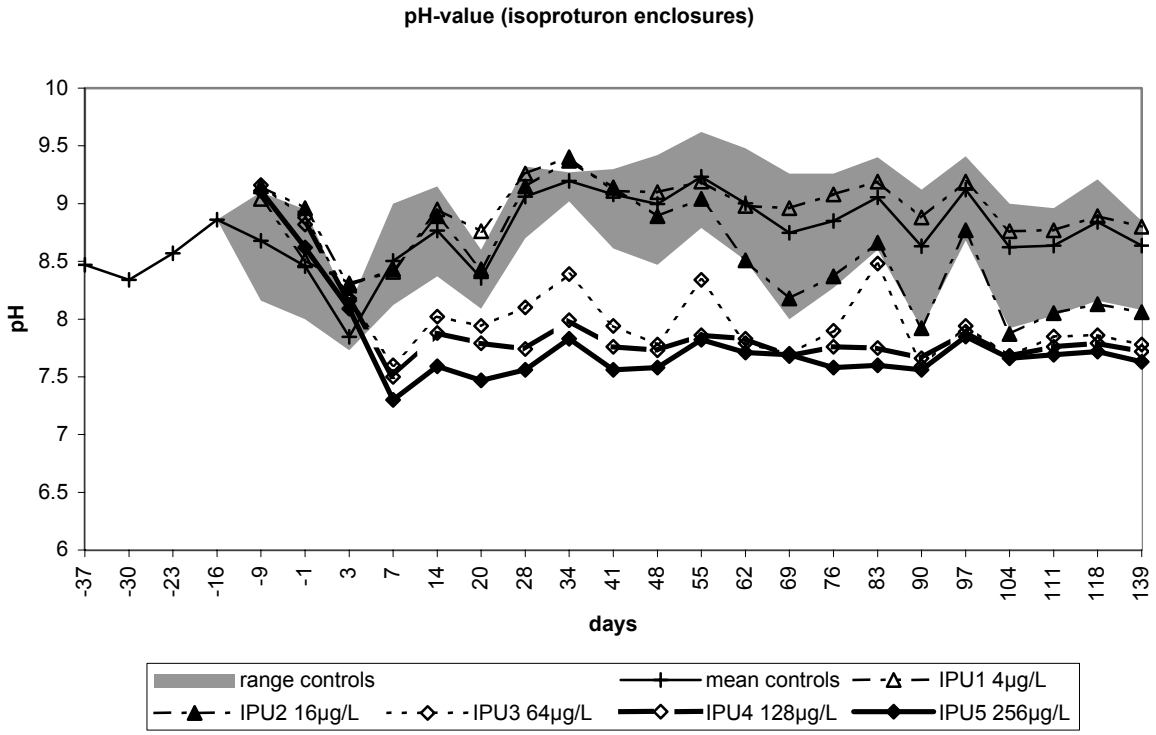


Figure 40: Development of the pH in the IPU study

No recovery could be demonstrated in the first year in all levels above the NOEC. IPU exerts a class 5 effect (BROCK *et al.* 2000 in EU 2002) on the pH.

4.4.5 Chlorophyll a

Contents of this pigment are given in Figure 41. The same precautions as in the CYP study have to be taken (cf. 3.4). Additionally, one has to be especially careful when trying to relate pigment amounts to cell numbers (i.e. abundance). ESER 2001 found increased amounts of the pigment per cell under IPU treatment in single species tests (beginning at 50 µg/L IPU). RIOBOO *et al.* 2002 saw a maximum in chlorophyll a at concentrations of 50 µg/L, too. The algae, having their photosynthesis apparatus blocked by IPU, try to compensate this by a kind of shade-type adaptation (FEDTKE 1974, LICHTENTHALER *et al.* 1980). More pigment does not

automatically equal more cells under these circumstances[14]. The method used here is particularly prone to these effects because it only takes the photosynthetic active pigment into account.

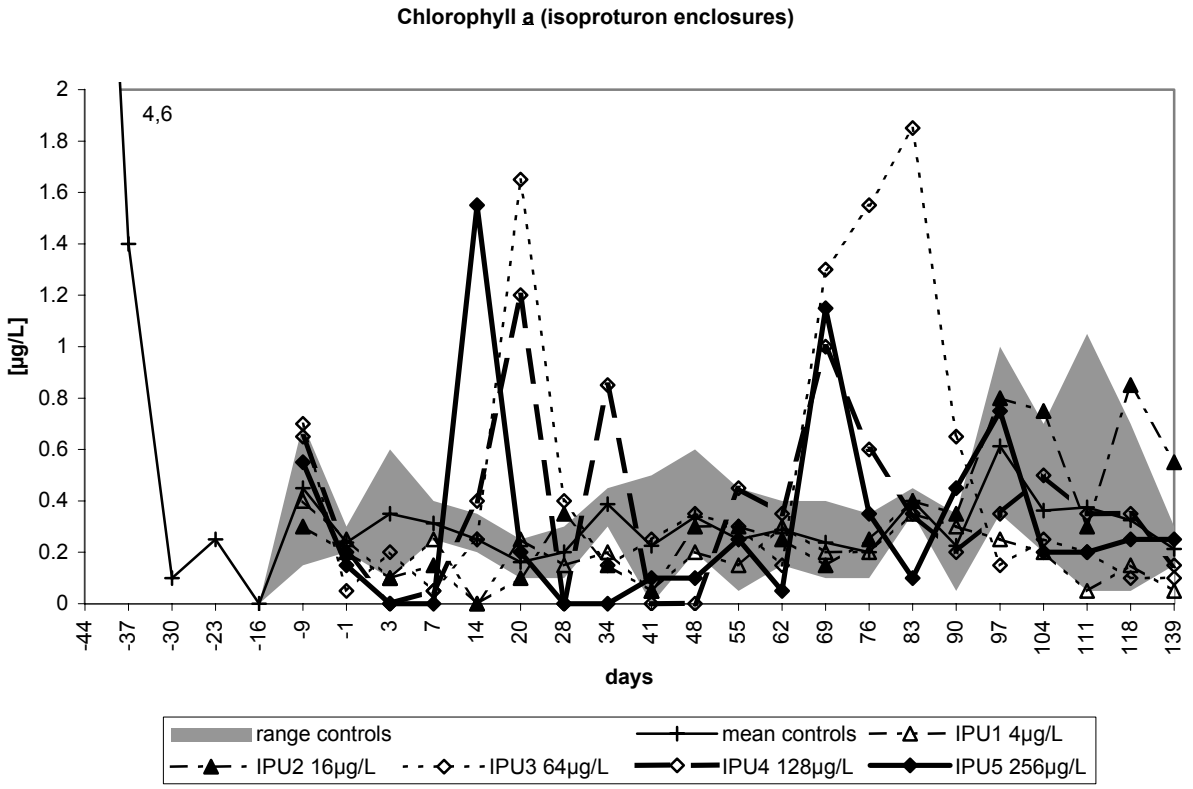


Figure 41: Chlorophyll a content in the IPU study

In this light interpretation of the curves in Figure 41 can be given in this way: In the first month a.t. more pigment is found in IPU3-5. Since there is no clear increase in the total abundance (4.6.2a), this may well be due to the "shade adaption". The later increase on day 69 a.t. can be seen in cell numbers to some extend as well (Figure 51, page 94). The development in IPU3 in chlorophyll a is exceeding it in height and time. Being later in the year (start of September), altered environmental conditions[15] may induce more chlorophyll a in the cells while still some amount of IPU is in the water (about 15 µg/L in IPU3).

All in all chlorophyll a shows only slight effects. Other parameters are more susceptible to IPU treatment.

[14] Although ESER 2001 found this connection as well, but discussed a kind of "blurred" correlation under IPU action.

[15] Especially light and temperature, or nutrition (see 4.4.7). Effects are thoroughly discussed in ESER 2001.

4.4.6 PRC analysis

The cdt values are displayed in Figure 42. This diagram is an almost ideal specimen of a dose-response pattern without recovery. Effects are present from day 14 a.t. until the end of the year.

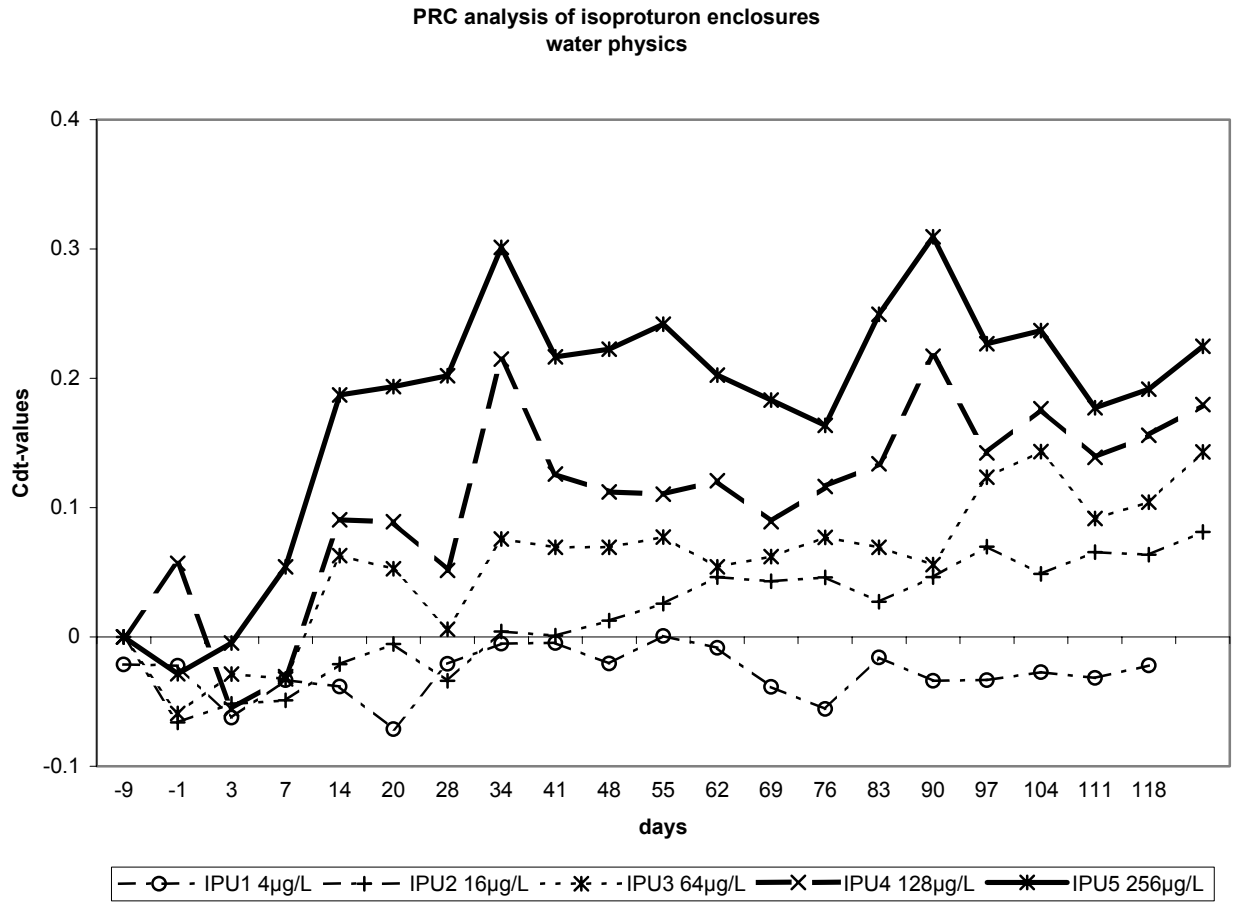


Figure 42: PRC analysis of the water quality parameters in the IPU study

This analysis is significant, p=0.005. It explains 49.3% of the variances by the treatment of which 65.7% are displayed. 40.4% of the variations are explained by the sampling day. Table 26 lists the "species" with absolute scores bigger than 0.5. PRC curves are rising with time. The parameters with scores lower than zero thus decrease with time while the others go in line with the curves.

Alkalinity and conductivity are increasing with treatment, pH and oxygen are falling. This is a well-defined DO-pH-alkalinity-conductivity syndrome (KERSTING and VAN DEN BRINK 1997). NEC calculations (90% regression coefficient) were possible for 8 sampling dates; lower, mean, and upper value are 7.45, 8.11, and 15.69 µg/L IPU, respectively. All these values are below 16 µg/L thus indicating a no observed effect level of 4 µg/L.

Table 26: "Species" scores of relevant water quality parameters (IPU study)

parameter	value
O_2 mg top	-0.72
O_2 mg bottom	-0.68
O_2 saturation top	-0.67
O_2 saturation bottom	-0.63
pH	-0.58
alkalinity	0.74
conductivity	0.74

4.4.7 Water chemistry

4.4.7a Overview

Summarized data of water chemistry are presented in Table 27. The test system is an oligo-mesotrophic one (SCHWOERBEL 1999). Parameters not influenced by the IPU treatment are in line with the controls and the CYP enclosures and are not presented here in greater detail. Compared to the natural waterbodies nearby the water is much softer. This is due to the growth of plants in the system without ample replenishing of calcium and/or magnesium ions from the sediment or any inflow. Especially submersed macrophytes bind ions in their bodies and thus the water is depleted. When the plants are degraded, e.g. because of the IPU treatment (Figure 48), an increase in the total hardness and the calcium ions is observed (see Figure 45 and Figure 47).

Table 27: Summary of water chemistry in the IPU study

		mean	std. dev.	min	max
TP [µg/L]	IPU-treated	27.86	7.66	7.23	46.98
	control	26.89	18.35	8.67	198.76
SRP [µg/L]	IPU-treated	6.72	4.44	0.00	21.84
	control	10.01	14.40	0.73	139.78
NO3-N [mg/L]	IPU-treated	0.07	0.10	0.01	0.65
	control	0.039	0.063	0.001	0.614
NH4-N [mg/L]	IPU-treated	0.08	0.07	0.01	0.73
	control	0.021	0.011	0.006	0.056
silicate [µg/L]	IPU-treated	659.56	362.03	26.88	1927.92
	control	454.95	368.30	29.32	1290.17
Na^+ [mg/L]	IPU-treated	3.31	0.39	2.48	4.52
	control	2.96	0.37	1.73	4.06
K^+ [mg/L]	IPU-treated	0.13	0.08	0.00	0.60
	control	0.06	0.04	0.00	0.22
Ca^{2+} [mg/L]	IPU-treated	17.33	6.84	9.38	35.40
	control	12.85	4.45	7.81	23.98
total hardness [°DH]	IPU-treated	4.89	1.13	3.30	8.20
	control	3.93	0.54	3.20	5.40

4.4.7b Silicate

The amount of silicate is inversely proportional to the diatoms' abundance in the waterbody (SCHWOERBEL 1999). In Figure 43 we can see an increase in this ion in the enclosures IPU3 (two dates) to IPU5 from day 28 a.t. onwards. For this reason, diatoms (either planktonic or periphyton ones) should be affected by the treatment.

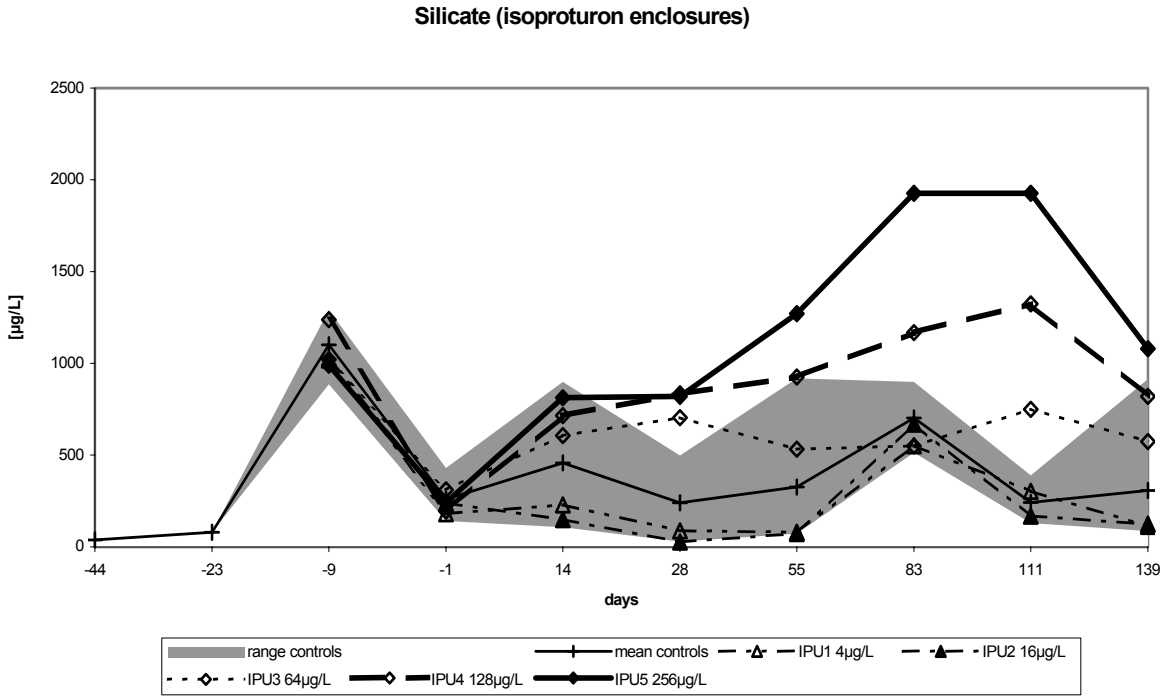


Figure 43: Silicate amounts in the IPU study

NEC was calculated on four sampling dates and gave values of 12.1 µg/L, 32.3 µg/L, and 109.7 µg/L. This result is in line with ESER 2001, who found a NEC (or NOEC in her words) of 17-62 µg/L IPU for the Bacillariophyceae and also saw increases in silicate (without calculating a NOEC).

4.4.7c Nitrogene compounds

NO_3-N showed an increase (0.1-0.6 mg/L) in IPU4 and IPU5 in the samples from day 83 a.t. onward (exceeding the control level, mean=0.04 mg/L). The controls contained more of this nitrogene fraction on day 139, too, but still less than the treated enclosures mentioned. Most probably this is due to decaying aquatic plants in autumn. Since macrophytes are negatively affected in IPU4 and 5 (4.5), the increase over the control level is supposed to be due to the same process (promoted by the herbicide). Oxygen is still present in the treated enclosures to enable the oxidation of ammonium to nitrate.

More intensely influenced is the NH_4-N. From day 28 to day 111 a.t. there are more than 0.4 mg/L in IPU5 (control mean 0.02±0.01 mg/L, see Table 27). Day 139 sees NH_4-N in line with the controls again. The value in IPU4 is higher than in the controls on day 28 (0.08 mg/L), 83 (0.09 mg/L), and 111 a.t. (0.09 mg/L). IPU3 has 0.07 mg/L (111 days a.t.). This may again be due to decaying plants.

4.4.7d Cations: Sodium, calcium, potassium

Sodium and calcium ions have a comparable development (Figure 44 and Figure 45).

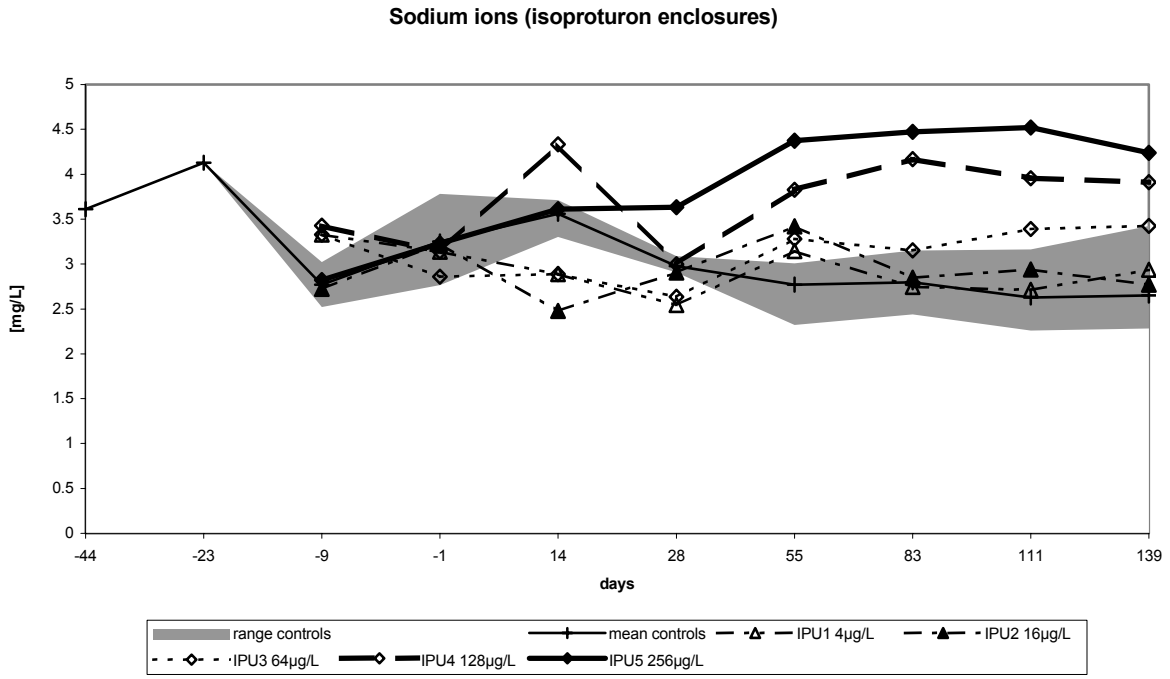


Figure 44: Sodium ions in the IPU study

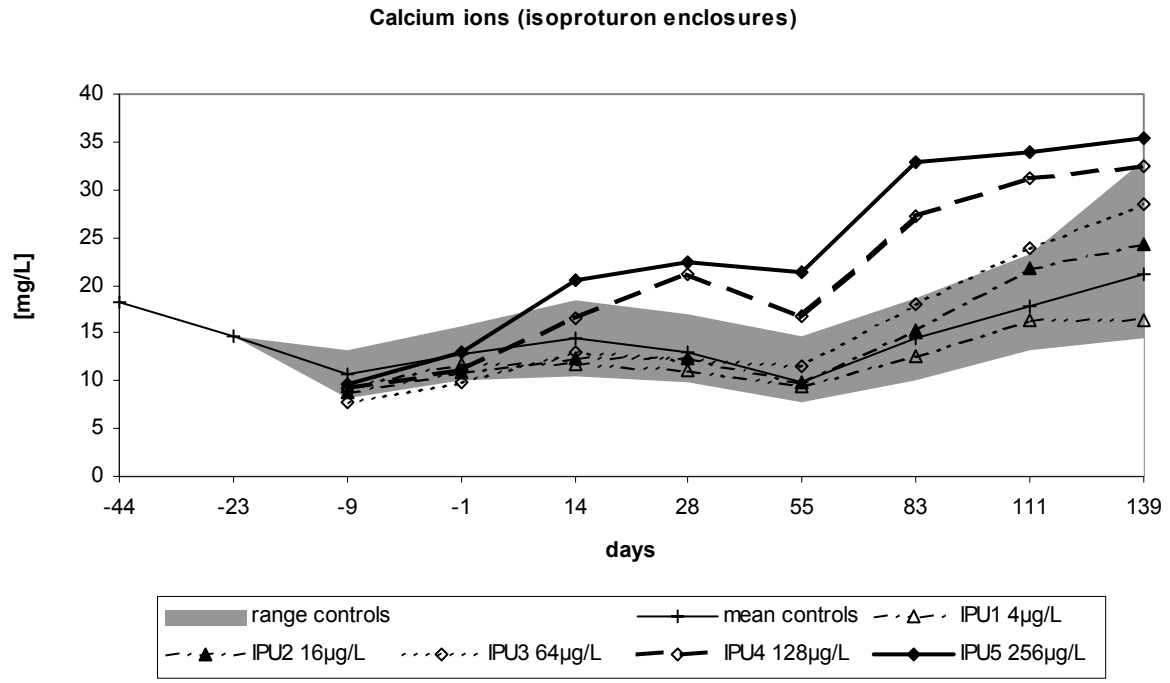


Figure 45: Calcium ions in the IPU study

A distinct increase with IPU concentration can be seen for day 28 a.t. onwards. This is almost certainly due to less photosynthesis and decaying plants. The same explanation can be given for potassium. The curves are somewhat different (Figure 46): IPU5 values are heavily increased from day 14 a.t. onwards; the values in IPU4 and 3 are higher than the controls

beginning with day 83 a.t.. All values except the ones of IPU5 are around the LOQ and must be dealt with care.

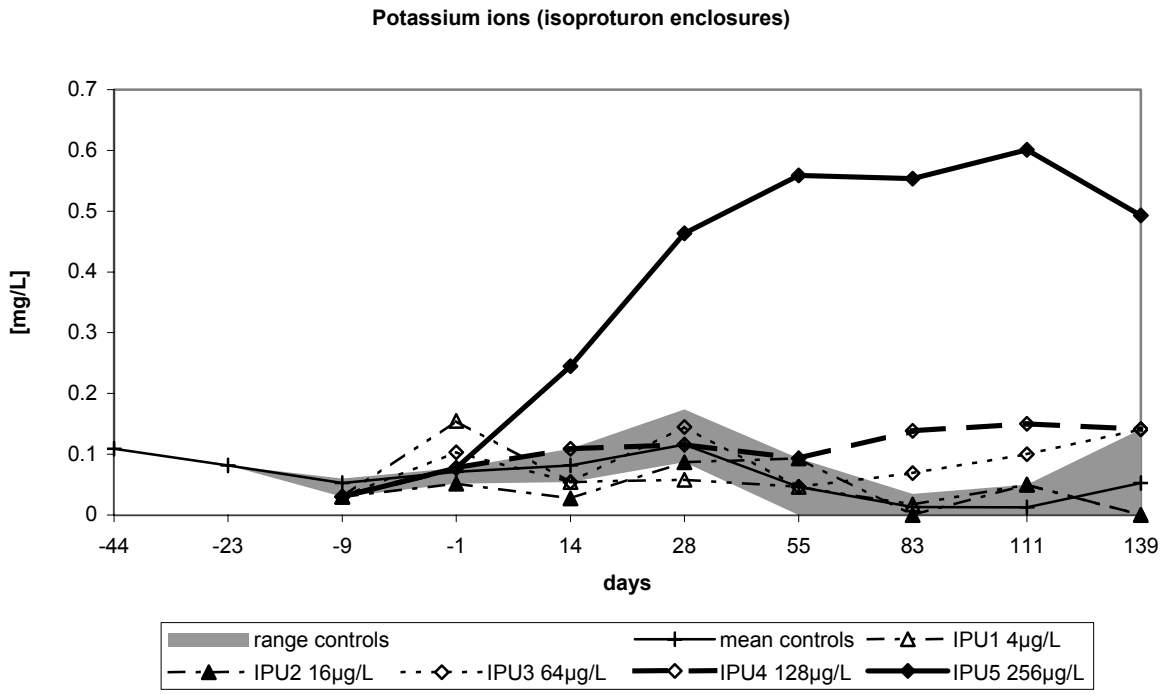


Figure 46: Potasium ions in the IPU study

NEC values for sodium and calcium parameters are comparable and to some extent lower than the IPU3 level (Table 28). The middle value is about 44 µg/L IPU. Potassium NEC is higher; approximately 80 µg/L. Regression was only possible on day 14, so this value is perhaps not valid, because the potassium amounts on that day were too low (LOQ=0.1 mg/L). On day 83 and 111 IPU3 values (64 µg/L) are also above the control level (Figure 46) but still very near the LOQ. So the NEC may be in line with the other ones. A LOEL of 64 µg/L might be addressed.

Table 28: NEC values cations in the IPU study

value [µg/L IPU]	NEC Na^+	N	NEC Ca^{2+}	N	NEC K^+	N
upper	62.05	5	82.05	5	100.93	1
middle	44.63	5	43.65	5	86.14	1
lower	33.70	5	25. 37	5	73.51	1

4.4.7e Total hardness

As stated above, hardness increases with treatment (Figure 47). IPU5 values are above the controls from day 14 a.t. on, those of IPU4 follow on day 28. IPU3 measurements exhibit slightly harder water on day 111 a.t.. NEC calculations gave (35.8 µg/L)-53.2 µg/L-(87.2 µg/L) (n=6). This, too, is indicating a LOEL of 64 µg/L (IPU3).

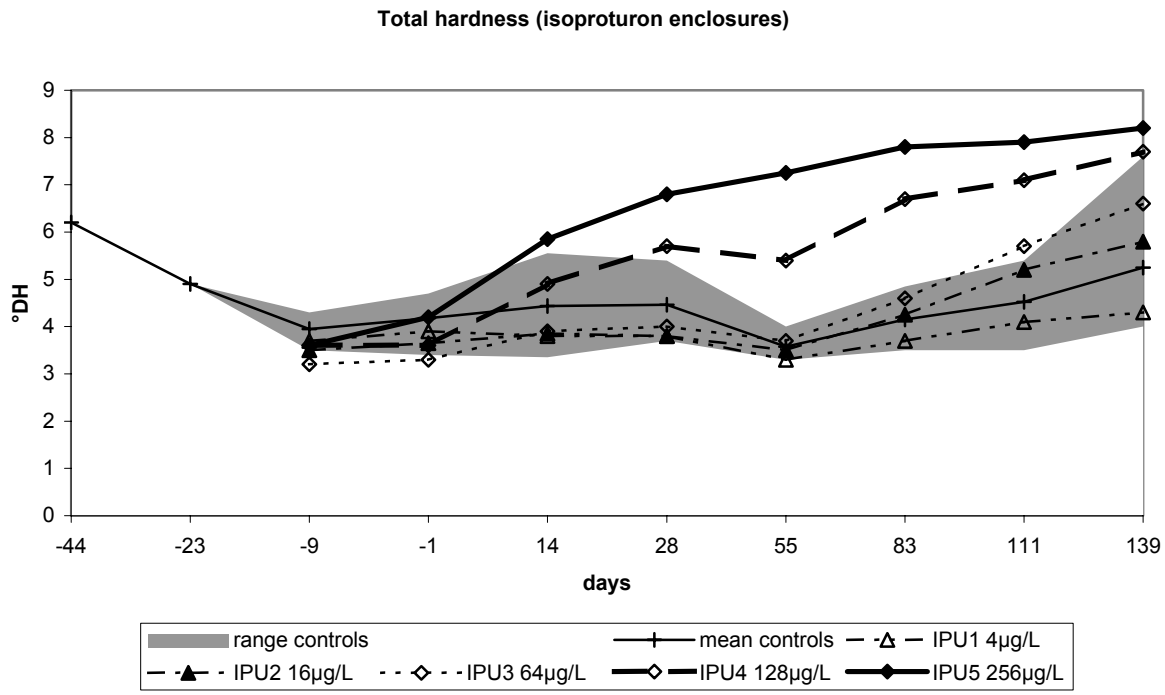


Figure 47: Total hardness in the IPU enclosures

4.4.8 Overview of treatment effects of IPU on the water quality

Water physical parameters (4.4.1-4.4.4) were clearly affected by IPU treatment. NOEC for all of them was 4 µg/L, that is lower than all the ones found by ESER 2001 in a comparable mesocosm. Recovery took about one year classifying them as class 5 effects (BROCK *et al.* 2000 in EU 2002). A pronounced DO-pH-alkalinity-conductivity syndrome (KERSTING and VAN DEN BRINK 1997) was found (see PRC analysis, 4.4.6). All these findings are corroborated by the outcomes of the study ESER 2001 conducted with IPU. The differences in the NOECs compared with this work stem for the method used calculating them. ESER 2001 used a procedure analogous to LIBER *et al.* 1992. In the presented study, the results of this method was also calculated but named "NEC"[16]. When comparing NEC to NOEC (Eser), data are matching. The value for them lie about 20-40µg/L IPU.

Chlorophyll a showed only minor effects induced by the treatment.

Water chemistry parameters were distinctly altered by 64 µg/L a.i. and more. The amount of ions in the water increased with treatment, particularly in the two highest concentrations. These findings are to some extend reflected in the NOEC for the macrophytes (4.5).

4.5 Macrophytes

The development of the macrophyte coverage in the IPU study is presented in Figure 48. Generally, in the controls and the three lower treated enclosures steadily more plants were

[16] No Effect Concentration, since they can reach any value for IPU and not only the ones realized in the experiment. Please note that exclusively NEC and the NOEC (sensu ESER) can be compared with each other.

growing. The value of the coverage started around 40% and was approximately 70% on the last sampling.

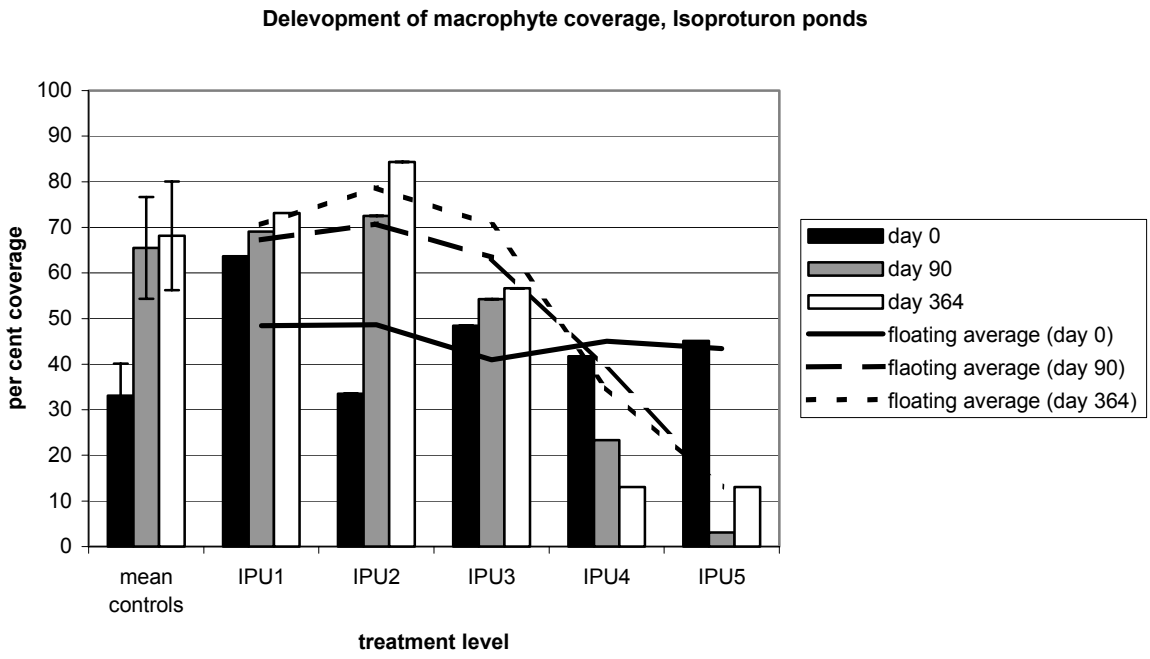


Figure 48: Development of the macrophytes under IPU influence

The opposite trend showed in the IPU4 and IPU5 enclosures (except the last value for IPU5).

Accordingly, IPU treatment had a distinct effect on this parameter. NOEC is 64 µg/L, which is a very reasonable one because the trend in growth changes in the higher levels.

ESER 2001 found only minor effects on the macrophytes beginning at 100 µg/L IPU in her study. This difference may be due to the macrophyte composition that was not the same. ESER had mostly *Potamogeton natans* in her ponds, whereas in the presented study the major plants were *P. lucens* and *Myriophyllum spicatum* (Figure 49). For the latter she also described more intense treatment effects backing the conclusion given above.

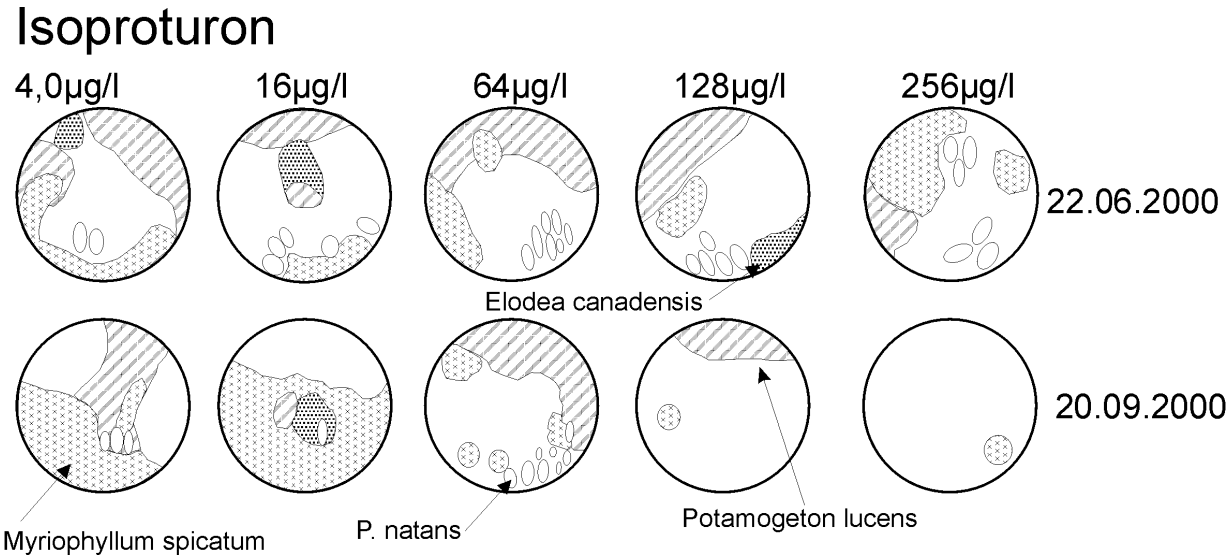


Figure 49: Macrophytes in the IPU enclosures in 2000

Increasing cover of the macrophytes in IPU4 and IPU5 is beginning in late June 2001, about one year after the treatment. IPU residues and/or environmental parameters enable growth from this point of time on. Most vigorous growth as well as most healthy looking plants were seen the higher the treatment level had been. This finding can help explain some of the effects seen in other endpoints of the study.

Changes in the macrophytes alter the microhabitat structure of the enclosures. BLINDOW *et al.* 2000 found that Cladocera that are associated to plant stands do not leave them. If the plants are affected by the treatment, the niche of these species is not present any more in the system. For example, zooplankton taxa found in the presented study are often associated to plants (LAWA 1996, e.g. *Chydorus sphaericus, Simocephalus vetulus*). COTTONIE *et al.* 2001 noted big differences in zooplankton communities of ponds dominated by either phytoplankton or macrophytes. The macrophytes cover was one of the most important control factors for zooplankton together with predation intensity in their study.

Effects of the reduced macrophytes cover on the zooplankton are therefore quite likely.

4.6 Phytoplankton

4.6.1 Composition of phytoplankton

The phytoplankton of the IPU study is dominated by only two species, *C. erosa et ovata* and *Ch. acuta* (Table 29), both cyrptophyceae. This is similar to the CYP study. Most of the other dominant species are present with comparable dominances as well. Therefore, a good comparison between the two studies is possible.

The phytoplankton biocoenosis was composed of the classes Bacillariophyceae (7 taxa), Chlorophyceae (52 taxa), Chrysophyceae (15 taxa), Conjugatophyceae (5 taxa), Cryptophyceae (3 taxa), Cyanophyceae (7 taxa), Dinophyceae (2 taxa), Euglenophyceae (6 taxa), Xantophyceae (2 taxa), and one Prasinophyceae.

Table 29: Dominat species in the phytoplankton (IPU study)

	species	dominance (IPU)
1	*Cryptomonas erosa et ovata* (Cryptophyceae)	29.3
2	*Chroomonas acuta* (Cryptophyceae)	23.9
3	*Nephroselmis olivacea* (Chlorophyceae)	4.3
4	colony forming Cyanophyceae	4.3
5	*Achnanthes minutissima* (Bacillariophyceae)	3.5
6	*Mallomonas sp.* (Chrysophyceae)	3.4
7	*Katablepharis ovalis* (Cryptophyceae)	3.3
8	Chlorophyceae ssp.	2.8
9	*Desmarella moniliformis* (Chrysophyceae)	2.4
10	*Monosiga varians* (Chrysophyceae)	2.2

The development of algal classes in IPU5 is presented in Figure 50. Treatment effects were concentration related, so the other enclosures showed a similar but less pronounced reaction. The development of the controls may be looked up in the CYP part (Figure 10, page 46).

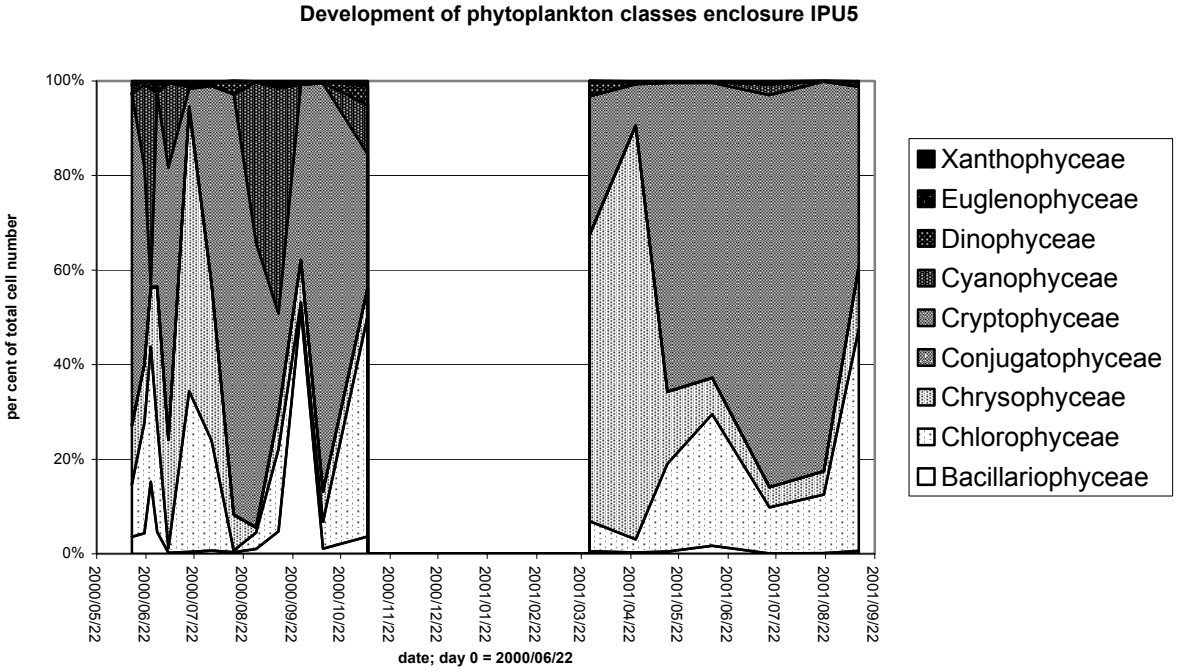


Figure 50: Development of phytoplankton classes in the IPU study

The treatment leads to big fluctuations in the class distribution in the first year, mainly in Chlorophyceae, Chrysophyceae, Conjugatophyceae, and Cyanophyceae. They all show rapid growth and a likewise fast decline shortly afterwards. The herbicide has clearly an influence on the algal composition.

In the second year, Chrysophyceae dominate in spring. This cannot be seen in the controls and is less pronounced the other treated enclosures. It may be due to the still relatively high amount of a.i. in the IPU5 enclosure in March and April 2001. It was about 14 µg/L and 9 µg/L for the first two samples in 2001, respectively. All other enclosures had an amount of less than 1.4 µg/L IPU. Thus, this maximum may be interpreted as the last of the fluctuations related to the treatment (see below). Further deviations are not too distinct and rather specific of the enclosure in consideration.

4.6.2 Abundance data

4.6.2a Total abundance

Figure 51 shows the development of the total numbers of phytonplankton cells in the IPU study in the first year. In the second year of the study, no effects were visible on this parameter. IPU amounts seem to be too low already to exert an effect (Table 24) or the biocoenosis has adopted to the herbicide by then.

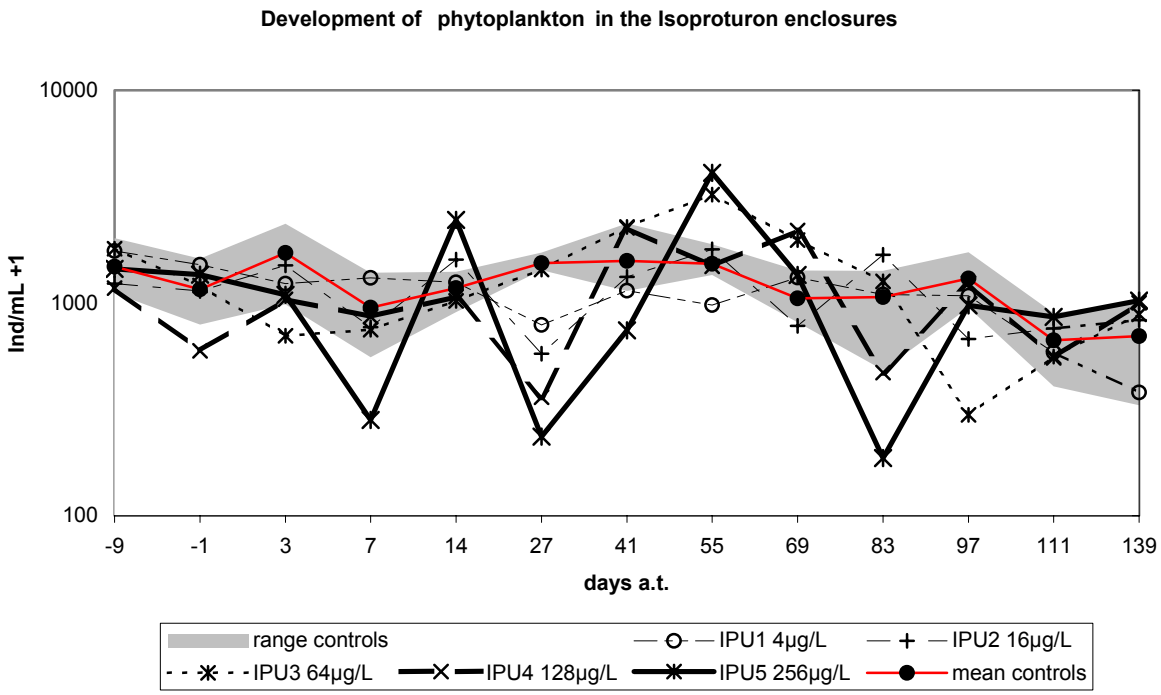


Figure 51: Total abundance of phytoplankton in the IPU enclosures

All treatment levels are outside the control range at least once. A distinct dose-response pattern cannot be seen. Rather there are fluctuations in cell numbers which increase with a higher amount of IPU in

1. amplitude and
2. speed (frequency).

IPU5 and IPU4 are affected most severely, IPU3 and IPU2 show the pattern in a quite comparable way, and IPU1 is merely somewhat "wavy".

Stating a clear NOEC is thus complicated. There are no identical values on two consecutive sampling dates except for IPU4 on day 7 and 14 a.t., but the direction changes: day 7 has a decline, day 14 a.t. an increase in algae. IPU1 and IPU2 levels are the NOEC on two different dates, again with the direction changing. Mean NEC value is 68 µg/L but naturally it has the same problems as NOEC. The curves (Figure 51) suggest no effects in IPU3 or IPU2. The higher value is backed by the NEC, the lower by the NOECs. ESER 2001 found clear effects on phytoplankton beginning generally with 40 µg/L. As a consequence, a no effect level between 16 µg/L and 64 µg/L is quite probable.

The fluctuating pattern is also seen in the class distribution of the algae (Figure 50). A connection to the oxygen contents is obvious, too. For example, when looking at the curve of IPU5 in Figure 39 on page 82, fluctuations can be seen as well. More algae were present in the enclosure on day 14 and day 55 a.t.. On these sampling dates, oxygen contents are higher than on those days when less algae could be found (day 7, 27, or 83 a.t.).

An explanation cannot be given without also regarding other parameters influencing the algal abundance, e.g. nutrition or grazing. A closer look at the "winners" and "losers" in the stated pattern is worthwhile, too. This will consequently be done in the following.

4.6.2b Species richness

The fluctuations seen in the total abundance may be due to the loss of some species. Their niche(s) may be taken by others, now being able to compete with the remaining species. This competition may eventually lead to major deviations in abundances over a short period of time. Additionally, slight changes in the environment of the algae may alter the outcome of the competition radically and thus lead to quick and thorough variations in cell numbers. Such variations in the abiotic environment are indeed seen after the treatment (see chapter 4.4). A simple cause-effect pattern cannot be expected, though.

The number of taxa in the IPU study is presented in Figure 52.

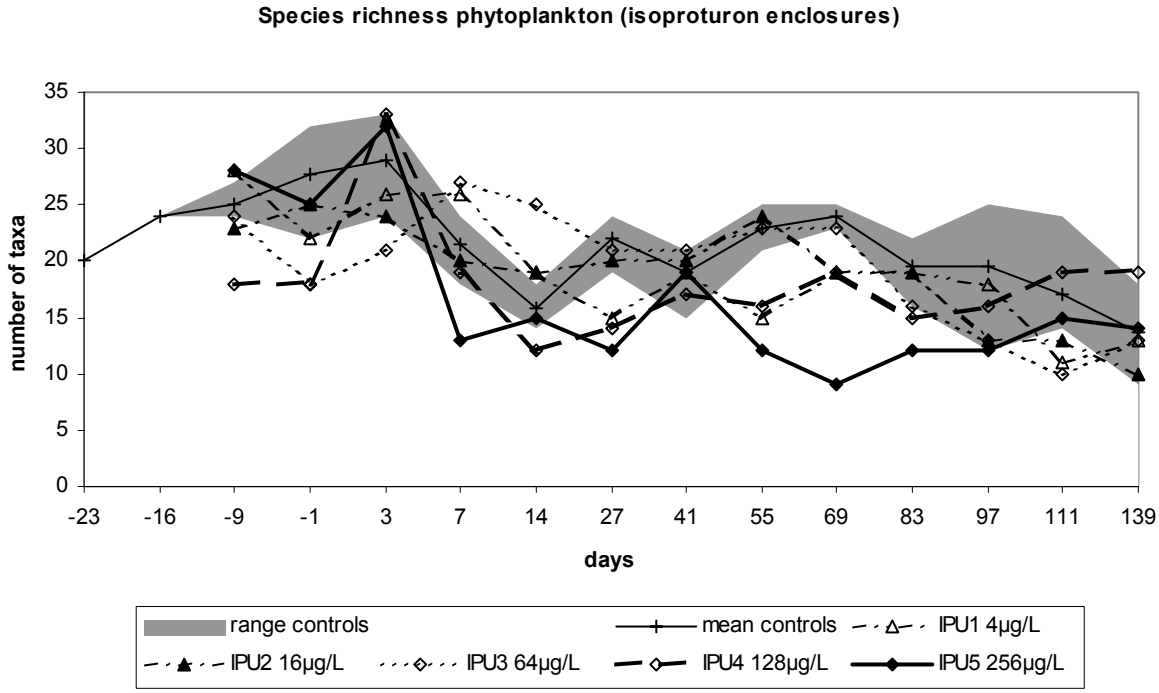


Figure 52: Species richness (taxa) in the phytoplankton of the IPU study

The number of taxa found is about 15 to 25 in the controls. There is a steady trend towards lower counts the later in the year the sampling took place. In the second year, numbers of species increase in autumn (data not shown).

Up to two weeks after the treatment IPU3 has even more taxa than the controls. In IPU4 and IPU5 less taxa were found up to day 97 a.t.. At least on some occasions IPU1 has lowered species richness, too. IPU2 is in the range of the controls most of the time.

NEC values are (39.0)-48.5-(65.1) µg/L (n=3), that means between IPU2 and IPU3. Recovery has taken place on day 97 a.t., resulting in a class 5 effect (BROCK *et al.* 2000 in EU 2002).

In species richness the fluctuating pattern of the total abundance cannot be seen (Figure 51). IPU treatment generally reduced the numbers of taxa found in the ponds. The increase in IPU3 shortly after the treatment may be due to better competitive conditions for taxa that had been below the detection limit of the microscopical analysis. Possibly they were more tolerant towards the herbicide than the better competitors in uninfluenced conditions.

4.6.2c Chroomonas acuta

The taxon investigated here is sensitive towards IPU (Figure 53). Abundances are clearly relating to the treatment between day 3 a.t. and day 55 a.t.. Williams' test indicated a NOEC of 16 µg/L ($p<0.05$). NEC is a lower, (0.2)-1.2-(24.5) µg/L (n=4). Excluding IPU5, the system has recovered on day 55 a.t. (class 3 effect, BROCK *et al.* 2000 in EU 2002).

IPU5 has very low abundance again on day 69 and day 83 a.t.. The curve progression is hinting at a fluctuating pattern like the one seen in the total abundance (Figure 51). At least on day 83 a.t. this algae is contributing to the pattern. Please keep in mind that it is the second most dominant species (Table 29). Obviously, direct toxic effects are not so severe any more at this point of time[17], so the fluctuations probably stem from secondary processes in the food web.

In the second year of the study, no treatment related effects were detectable.

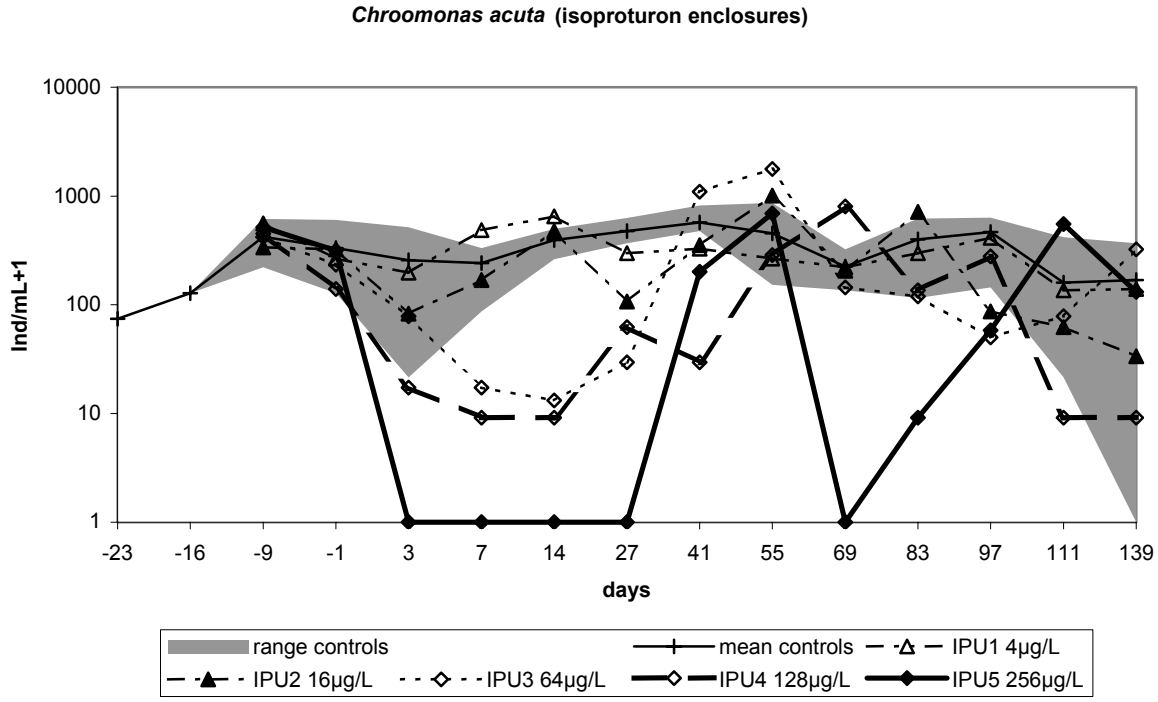


Figure 53: Development of *Chroomonas acuta* in the IPU study

4.6.2d Cryptomonas erosa et ovata

This taxon is the most dominant one in the study (Table 29). Effects of the herbicide on it are presented in Figure 54.

In the first year of the study, abundances are affected by the treatment. A dose-response pattern is seen up to day 14 a.t. (excluding IPU5). The algae's NEC in this time slot is (3.3)-5.9-(11.1) µg/L IPU (n=2), the NOEC 16 µg/L. The NOEC was found on two consecutive dates and is thus employed for the whole study.

From day 14 on the fluctuating pattern re-appears (treatment related, see also the total phytoplankton abundance, 4.6.2a). In IPU5 it already starts on day 7 a.t.. All the oscillating

[17] About 70 µg/L IPU are still present in IPU5 on day 83 a.t..

curves reach control level again on day 97 a.t. (with minor exceptions). A separate NOEC or NEC cannot be given.

Again, the fluctuating pattern may be a secondary effect. IPU treatment clearly affects the taxon's abundance (see also 4.7.4).

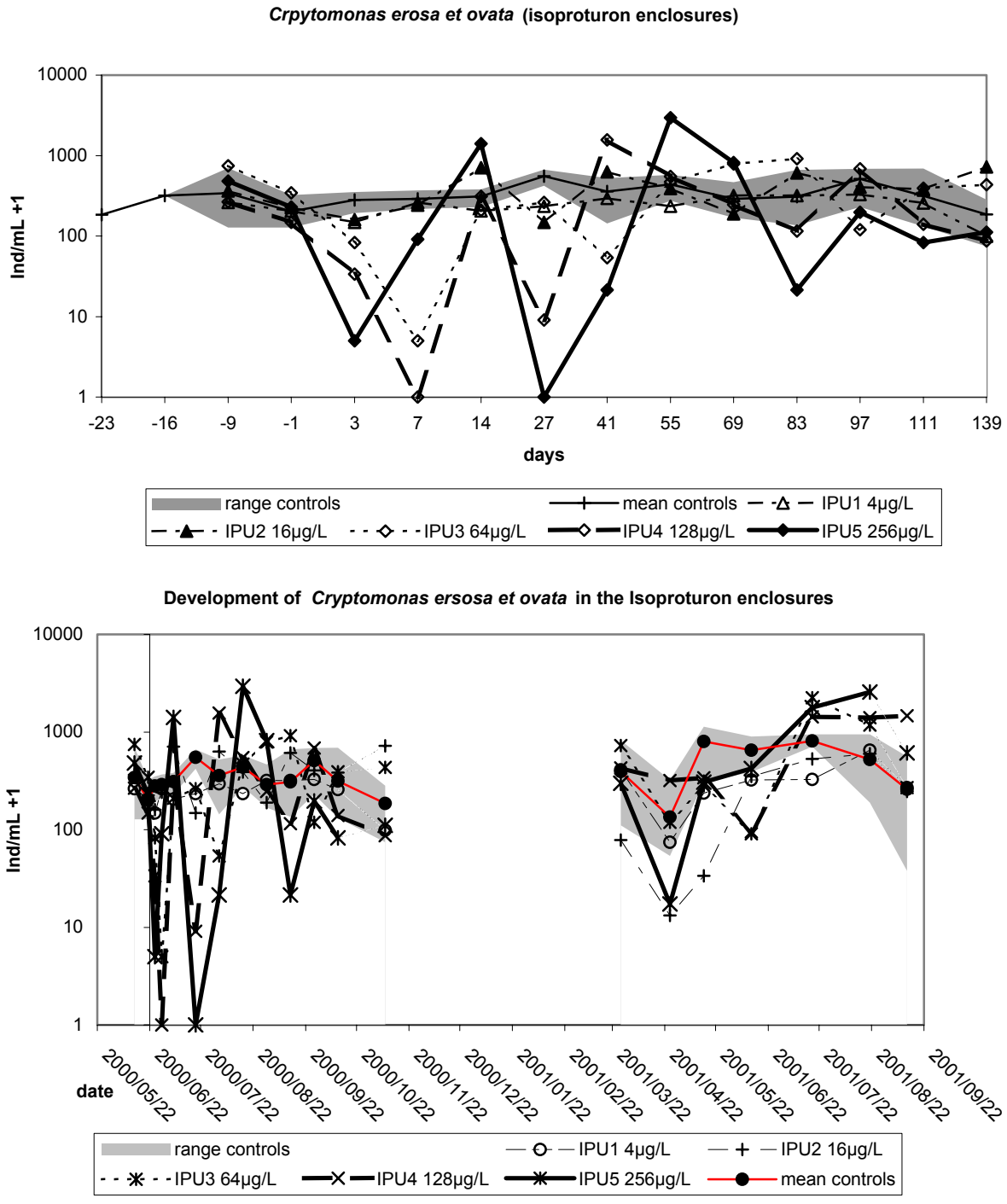


Figure 54: *Cryptomonas ssp.* in the IPU study; top: year one, bottom: both years

Early in 2001 differences in the abundances are still present but not clearly related to the treatment level. The situation changes in July, when IPU3 to IPU5 have higher numbers of the taxon than the controls; this time in line with the amount of herbicide applied. The NOEC for

this increase is 16 µg/L (IPU2), which is corroborated by the NEC: (5.8)-12.8-(28.4) µg/L (n=1).

This development is corresponding to

- the detoxification of IPU (4.1);
- the amount of macrophytes in the enclosures (4.5).

When no herbicide is hindering growth and competition for nutrition/light etc. is diminished, more algae are found in the ponds. This is a pronounced secondary effect.

4.6.2e Bacillariophyceae

Variation due to the herbicide are expected for this family because of the development in silicate (4.4.7b). Data is presented in Figure 55.

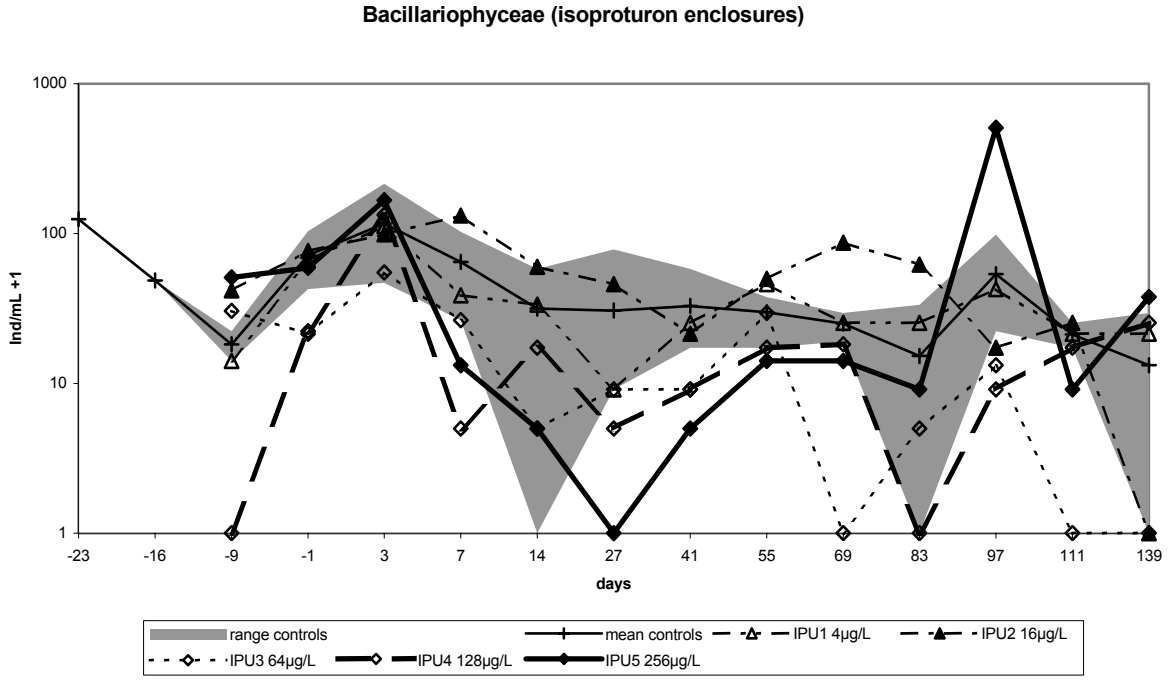


Figure 55: Development of bacillariopyhceae in the IPU study

Abundance in the controls is quite low; on days 14 and 83 a.t. no planktonic diatom at all was found in at least one of the control enclosures. In the second year of the study, even less of these algae were found. Consequently, analysis in this year did not give satisfactory results.

The curves in Figure 55 are suggesting an effect in IPU4 and IPU5, sometimes also in IPU3. NEC is backing this interpretation, (18.8)-38.5-(90.0) µg/L IPU (n=3). ESER 2001 found planktonic diatoms affected by IPU at concentrations of ≥20 µg/L a.i. using pigment analysis alone. This finding is quite consistent with the somewhat higher NEC found here using a direct method of sampling (not via the amount of pigments). Pigment contents may well be altered at sublethal concentrations (see 4.4.5 for a discussion). These values are corroborated by the findings in silicate (4.4.7b), where effects also started in IPU3 (64 mg/L a.i.) on some occasions.

Unfortunately, this is where the similarities end. Effects in silicate are relatively pronounced for the two highest treatment levels. Such a development cannot be found here. On day 97 a.t.

IPU5 even sees a maximum in diatoms. From day 55 to the end of the year numbers of Bacillariophyceae in IPU4 are just slightly lower than in the controls. The effects on the silicate content may therefore be due to severe changes in the diatoms' periphytic community. In fact, ESER 2001 noted a LOEC of 80 µg/L IPU for this community, a value that matches well with the effects in silicate in the presented study.

4.6.2f Chlorophyceae

The reaction of the Chlorophyceae is shown in Figure 56. All the taxa of this class had a more or less similar development under IPU influence (data not shown).

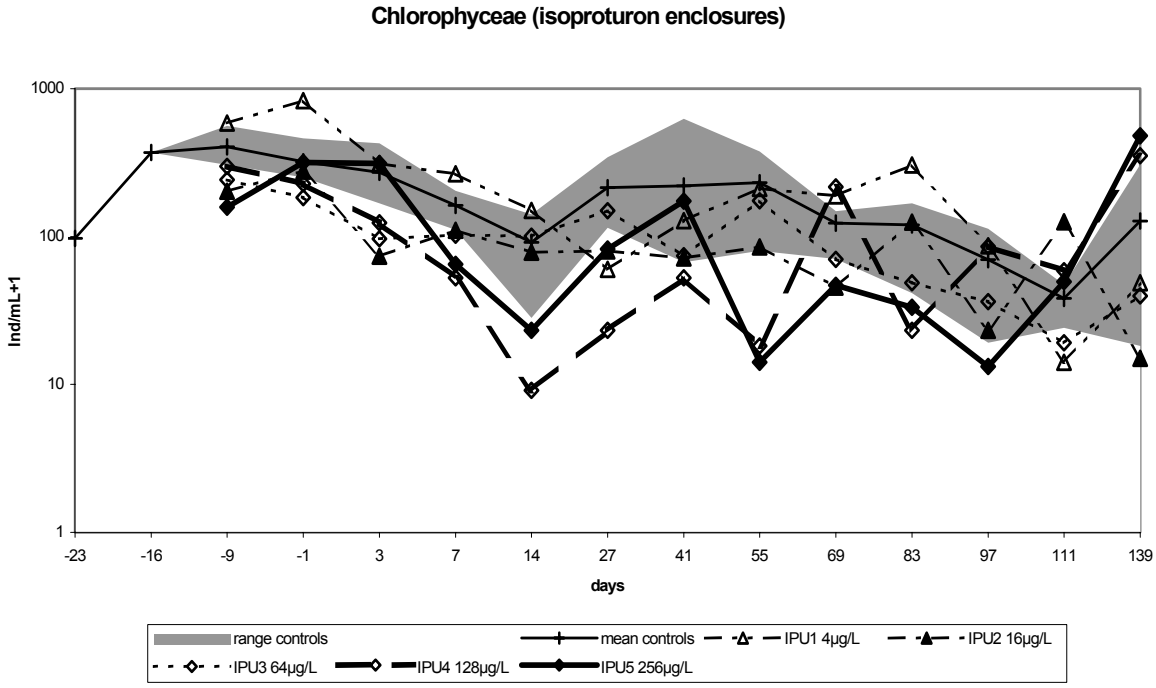


Figure 56: Chlorophyceae in the IPU enclosures

Well-defined effects are visible in IPU4 and IPU5. Williams' test was able to define a NOEC (decrease under IPU influence) from day 7 a.t. to day 97 a.t.. It is 64 µg/L IPU. Additionally, the fluctuating pattern found in other taxa can be seen here as well. Note that IPU5 has a higher abundance than IPU4 until day 55 a.t.. Accordingly, some kind of secondary influence plays a role for the development of these algae, too. Recovery may be noted on day 97 a.t..

The NEC is 43.1 µg/L (n=4, mean lower value). The other NEC data was much too high to be plausible. Both the NEC and the NOEC are corroborated by the findings of ESER 2001, who noted a value of 40 µg/L and higher of the Chlorophyceae.

In the second year of the study, no effects of the treatment could be detected.

4.6.2g *Nephroselmis olivacea*

The most sensitive species against IPU was *Nephroselmis olivacea*, a member of the Chlorophyceae. NOEC for this taxon was 4 µg/L IPU, a value that was found on five consecutive dates. Variations in the abundances were fairly high, so NEC calculations were not successful (bad regression, implausible high or low values). The curves show a distinct decline in cell numbers in all ponds but IPU1 (Figure 57) in 2000.

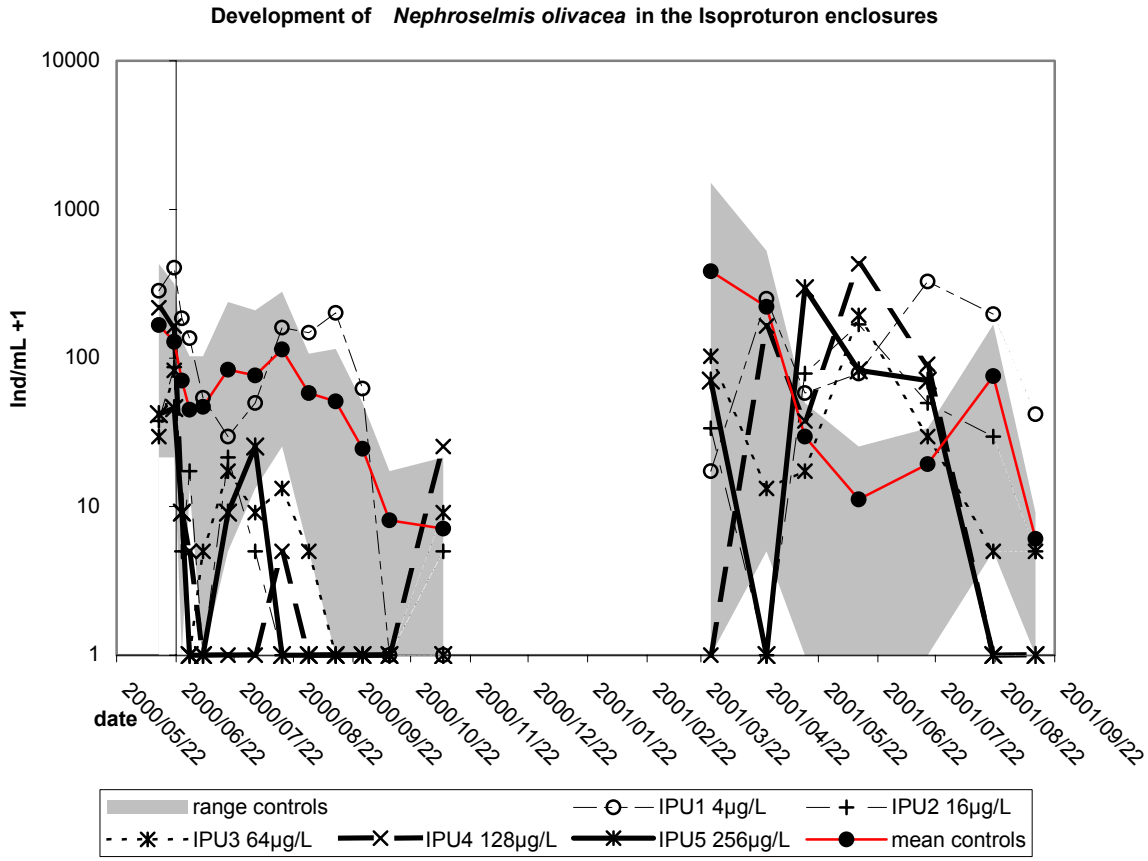


Figure 57: Development of *Nephroselmis olivacea* in the IPU study

In 2001, cell numbers in all treated enclosures lie above control level from late May to July; in other words around the time when all the herbicide is lost from the water column. *N. olivacea* is a small algae and may reach high numbers quite fast when conditions are good for it ("r" strategy, see SCHWOERBEL 1999, SOMMER 1994). The process is slowest in IPU1, where the macrophytes were not influenced by the treatment. The fastest increase is seen in IPU5. As a result, together with the high sensitivity of the species, this must be a secondary process again.

Normally, the maximum of small green algae can be expected in spring, when growing conditions are good for them and zooplankton is not able the reduce their numbers (SOMMER 1994). This is hinted at in the development of the controls. The treated enclosures have their maximum in these algae in summer, when normally bigger algae are dominating the phytoplankton community (SOMMER 1994). The increase is statistically significant, too ($p<0.05$ in Williams' tests). A NOEC smaller than 4 µg/L can be noted for this delayed development.

The treatment had a long lasting impact on this species (class 5 effect, BROCK *et al.* 2000 in EU 2002).

4.6.2h Chrysophyceae

The effects of the treatment on Chrysophyceae are presented graphically in Figure 58. No pronounced impact is visible in the first year. There is a maximum in IPU3 on day 27 a.t. and a minimum in IPU5 on day 69 and 83 a.t.. NOEC calculation indicated changes in the direction of the reaction towards IPU, either an increase or a decline. The lowest value is 16 µg/L (days 41 and 55 a.t.). NEC is in this range with 18.1 µg/l-23.4 µg/L-153.2 µg/L (n=3) for the whole study.

Reactions are therefore more probably of an indirect nature. The fluctuating pattern discussed in the above paragraphs is somewhat reflected in the changed direction of the IPU influence but may not be so directly linked to the herbicide as in the taxa above.

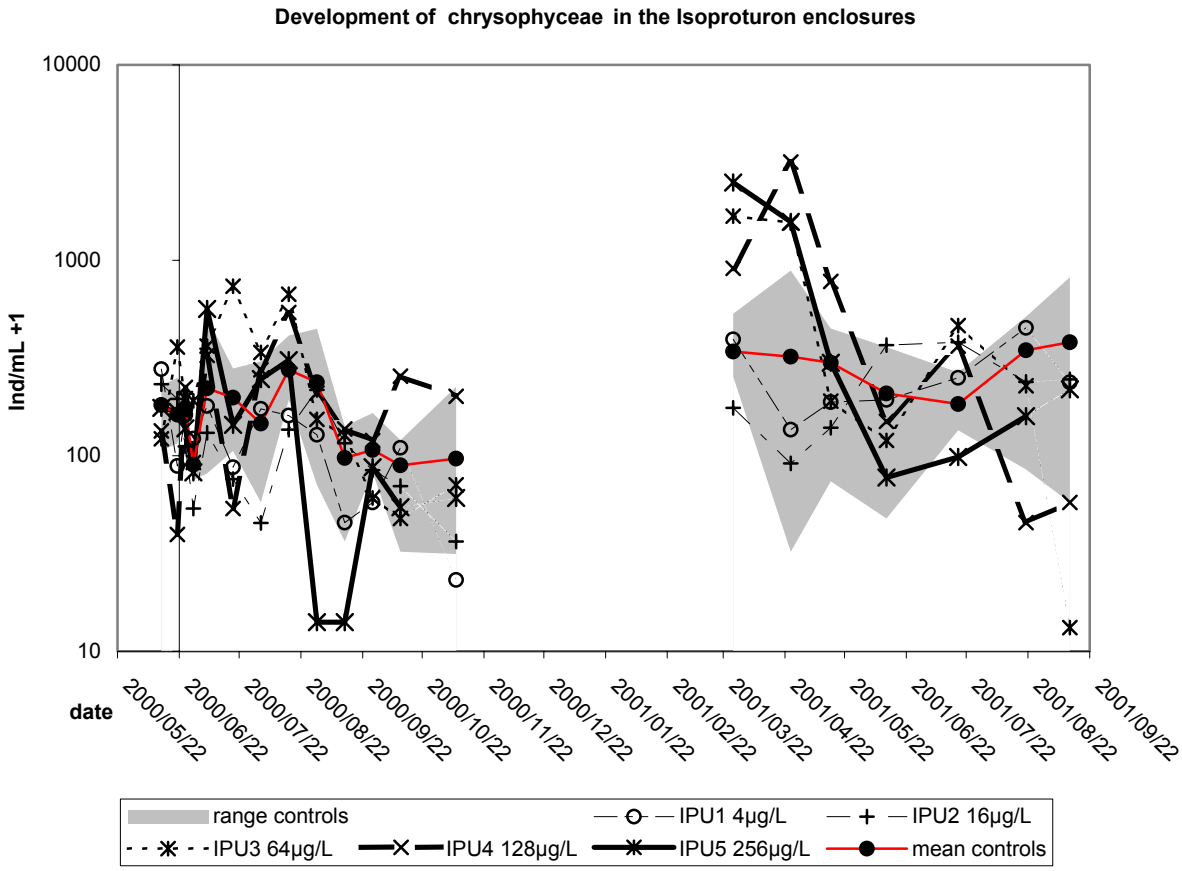


Figure 58: Development of the Chrysophyceae in the IPU study

In the second year of the study, IPU related deviations result in a spring increase in IPU3 to IPU5. NOEC is 16 µg/L again which can be proven by looking at the curves. They showed no effects in IPU2. This increase in Chrysophyceae is also seen in the relative dominance of the algae's classes (Figure 50).

These late reactions clearly suggest secondary effects of IPU on the Chrysophyceae. Competitive interactions under the influence of, among others, IPU concentrations (4.1), pH (4.4.4), oxygen (4.4.3), macrophytes (4.5), and other algae (especially 4.6.2d and 4.6.2e) allow

the Chrysophyceae to grow to higher numbers. When the parameters mentioned above are in the control range again, Chrysophyceae do the same.

4.6.3 Community analysis

4.6.3a Shannon index and evenness

Mean evenness is 0.65 in the IPU enclosures, and 0.66 in the controls. Shannon index shows a higher deviation: It is 1.89 in the IPU ponds and 2.03 in the controls. The evenness is presented graphically in Figure 59.

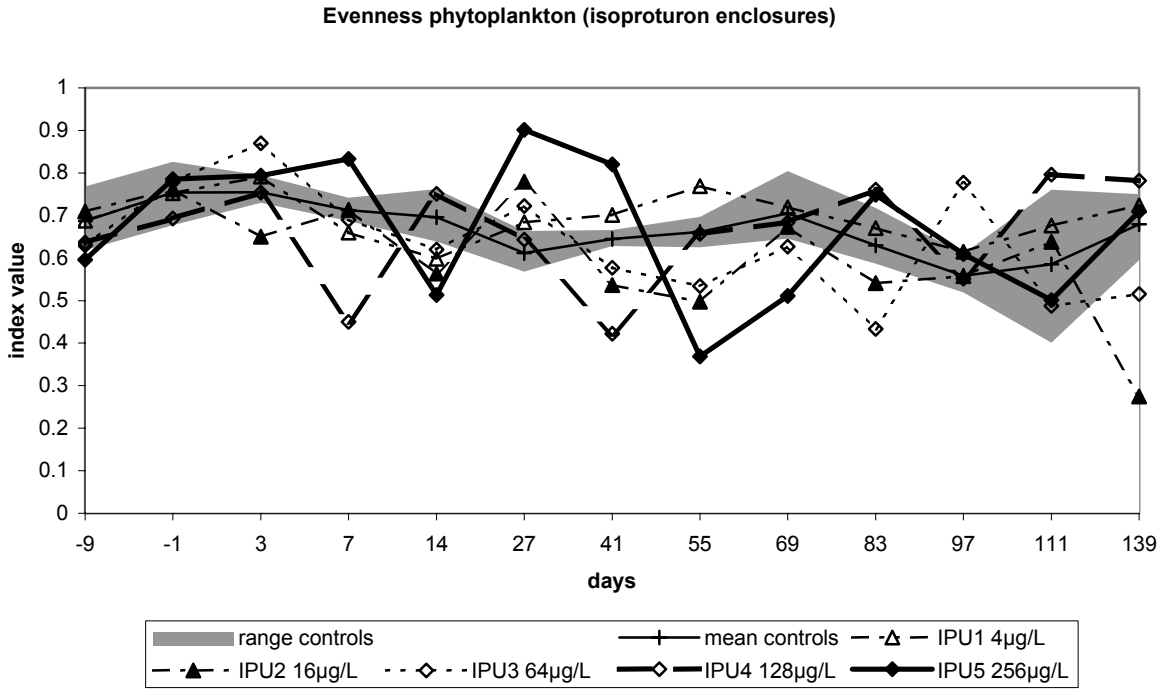


Figure 59: Evenness of the phytoplankton in the IPU study

NEC could only be calculated on day 3. Data is listed in Table 30.

Table 30: NEC in µg/L of Shannon index and evenness in the phytoplankton (IPU study)

NEC [µg/L]	Shannon	Evenness
lower	38.1	46.8
middle	68.2	93.6
upper	122.2	187.1

IPU4 and IPU5 show distinct effects of the herbicide, exhibiting a fluctuating pattern again. All the other enclosures are more or less in line with the controls or not clearly affected by the treatment. For example, IPU3 is between IPU1 and IPU2 for some time (days 14 to 55 a.t.). A minor disturbance may be noted. Values for the NEC back this interpretation. They are all similar to concentrations of IPU3.

4.6.3b RAD index

RAD index definitely reacts to the treatment (and thus the algae), see Figure 60. The fluctuating pattern found in earlier discussed analyses is represented here by the quite high number of peaks in the curves. This is especially evident in IPU5 to IPU3.

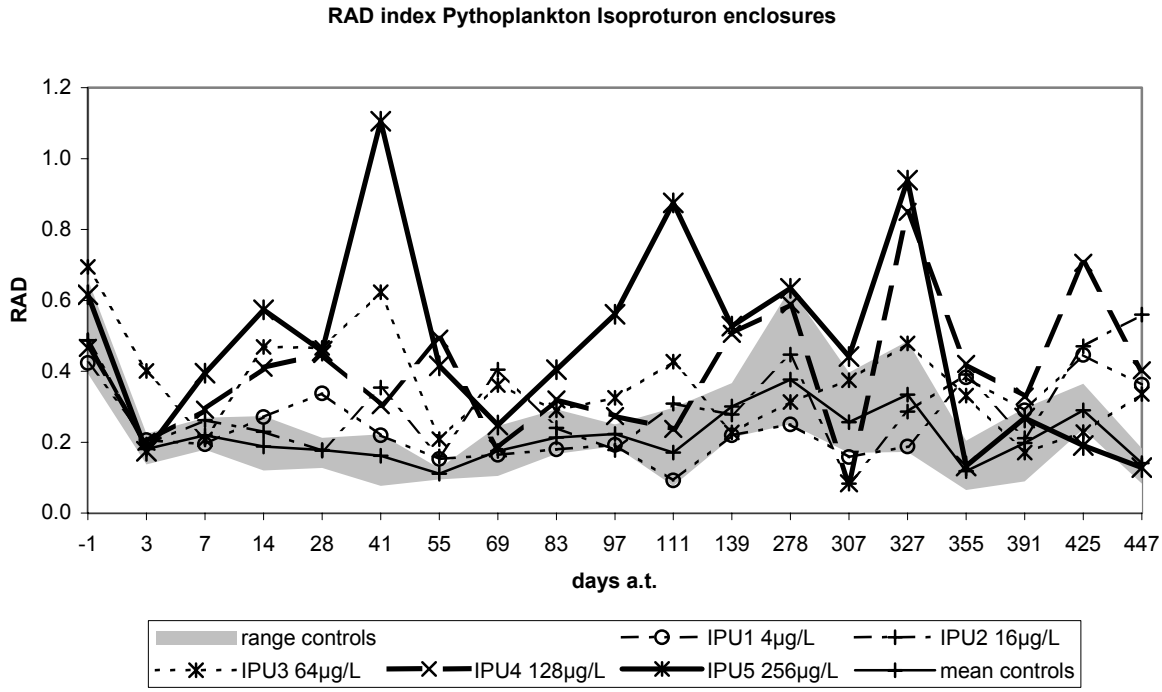


Figure 60: RAD index of the phytoplankton in the IPU study

NEC in the first year was 17.4-33.1-63.4 µg/L, n=2. IPU3 is repeatedly higher than IPU4, thus hindering regression.

In spring 2001 (days 278 and 307 a.t.), the control range gets wider. Due to this development, the treated enclosures do not show deviations any more. Later on, the impact gets more evident again but is not treatment related any longer. IPU5 is in line with the controls from day 355 on, and IPU4 shows the biggest dissimilarity to them. A disturbance of the system is still present but altered by secondary interactions that distort the treatment relation.

Above mentioned impacts and values suggest a no effect level of 16 µg/L or even a little bit higher. Secondary effects alter the pattern of the reaction of this index, too.

4.6.3c PRC analysis

Cdt values are plotted against time in Figure 61. Up to day 41 a.t. a dose-response pattern is obvious. IPU4 and IPU5 had almost the same effects on the community. Later on, only IPU5 shows bigger deviations. The other enclosures are not sorted by their herbicide concentration and their curves are rather noisy. This is hinting at vague disturbances as caused by secondary interactions.

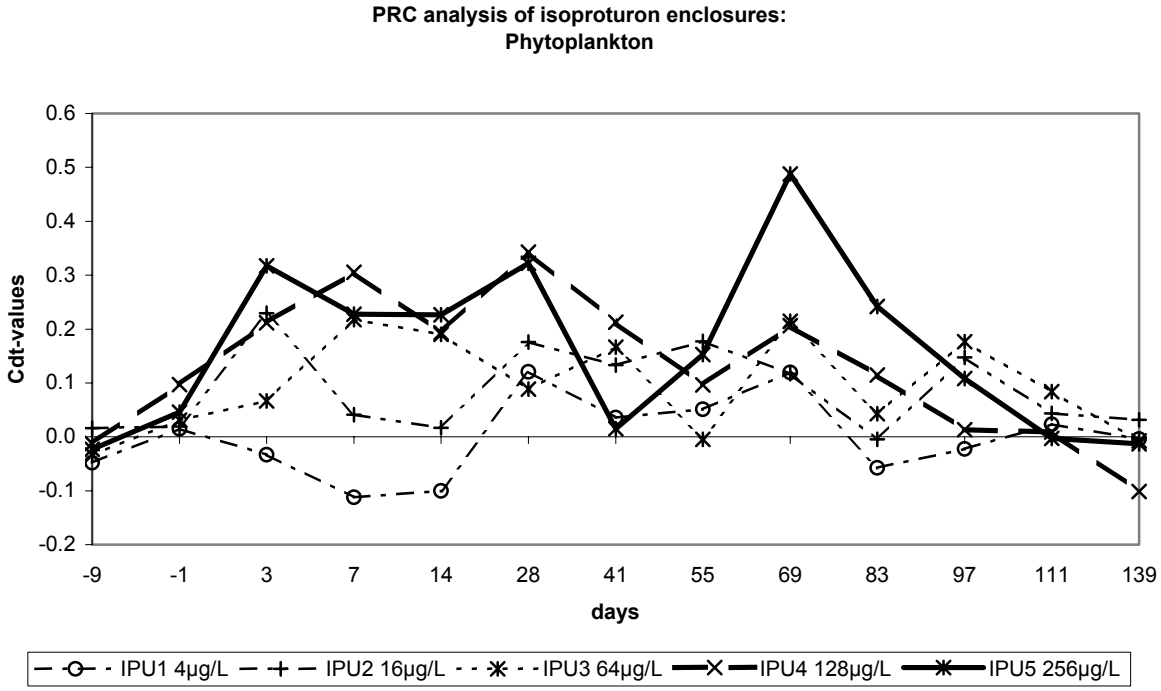


Figure 61: PRC analysis of the phytoplankton in the IPU study

This analysis is significant, p=0.01. It explains 46.2% of the variances by the treatment of which 12.5% are displayed. 28.1% of the variations are explained by the sampling day. Species scores are listed in Table 31.

Table 31: Phytoplankton taxa with a PRC species score >0.5 (absolute value)

taxon	score
Chroomoans acuta (Cryptophyceae)	-1.17
Nephroselmis olivacea (Chlorophyceae)	-1.11
Cryptomonas ssp. (Cryptophyceae)	-0.53
Coelastrum sp. (Chlorophyceae)	-0.52
Monosiga varians (Chrysophyceae)	-0.52
Gomphosphaeridoideae (Cyanophyceae)	0.63
Cyanophyceae	0.79

As shown above, *Chr. acuta* and *N. olivacea* are very sensitive towards IPU. Their scores below zero together with the rising curves clearly indicate this behavior. *Cryptomonas ssp*. are found to be sensitive, too, in spite of the fluctuating pattern of their abundance (4.6.2d). Blue-green algae are enhanced by the treatment. This finding is corroborated by the data discussed in 4.6.1, Figure 50.

A dose-response pattern is visible up to day 41 a.t.. This is the point of time at which *Chr. acuta* has recovered from direct treatment effects.

As already seen in the CYP study, PRC is not capable of resolving secondary effects if they have no linear reaction to the treatment regime. Quite obviously, secondary effects do not show this behavior in general (and they do not show it in the study presented, too). Effects linked to competition and the food web seem to get more important with time (see abundance data analyses, 4.6.2), so PRC is not the best tool of investigating the IPU data after day 41 a.t..

$NOEC_{community}$ calculations lead to a value of 16 µg/L (days 7 to 28 a.t.), corroborated by the NEC: 7.6-12.6-15.7 µg/L (n=4, for all cdt values).

4.6.4 Overview of treatment effects of IPU on the phytoplankton

NOECs for some taxa and parameters are listed in Table 32. The lowest value is the 4 µg/L for *N. olivacea*. This Chlorophyceae was the third most dominate taxon in the study (Table 29).

Table 32: Summarized NOECs of phytoplankton in the IPU study

taxon	NOEC [µg/L]	direction of IPU influence on data /remarks
Chlorophyceae	64	
Chroomonas acuta (Cryptophyceae)	16	
Chrysophyceae	16	
Cryptomonas erosa et ovata (cyrptophyceae)	16 (the same for both years)	sensitive in the beginning, then fluctuating pattern, increase with IPU in the second year
Cyanophyceae	n.n.	
Desmarella moniliformis (Chrysophyceae)	no pronounced effects	
Monosiga varians (Chrysophyceae)	128	
Nephroselmis olivacea (Chlorophyceae)	4 or lower	very sensitive, secondary increase in the second year of the study
total abundance	not clear, maybe between 16 µg/L and 64 µg/L	fluctuating pattern
$NOEC_{community}$	16 µg/L	

Algae in enclosures with the treatment level of 64 µg/L IPU (IPU3 enclosure) and above were often affected. Cyanophyceae took advantage of the treatment, statistically proven only by the PRC's species scores. Direct effects of the herbicide were frequently combined with secondary ones (see below). Total abundance, *Chroomonas acuta*, and *Cryptomonas ssp.* as well as species richness and *Nephroselmis olivacea* were most sensitive to the herbicide directly.

Excluding the latter two endpoints, the algae proceeded to a fluctuating pattern suggesting major changes in the algal community structure because the effects occurred in the two most dominant species (Table 29). This can be explained by an adaptation to the new "environmental" parameter, the IPU concentration. Because the herbicide gets less and less with time, the algae's environment changes constantly. The variations in the physicochemical parameters (4.4) as well as the macrophytes (4.5) must be kept in mind here, too. Fluctuations may be a consequence (or the reason) of changes there, too. Moreover, this pattern is linked to the zooplankton (4.7). Possible interactions are discussed there in greater detail.

Secondary effects (i.e. fluctuations) were also frequently noted in other taxa. They were too unspecific and linked to too many variables to find a clear-cut cause-and-effect chain (or even the main factor). Too little is know about specific interaction(s) of nutrition, light, macrophytes, etc., and the algae in question. Possible interactions causing a decline or increase of an endpoint were given above with the taxa data.

Some treatment effects were still visible in the second year. Deviations lasted up to one year after the treatment or they started occurring then. This time represented the complete degradation of IPU residues in the water column (4.1). Changes in the macrophytes (4.5) helped explain different pattern of reactions as well. For example, *Cryptomonas ssp*. increased after the onset of June 2001, when IPU was finally degraded. Additionally, an increase in Chrysophyeceae beginning in spring 2001 was in line with the controls again at that point of time. Both deviations started at a concentration of 64 µg/L (IPU3). These effects were most probably secondary ones again. This also holds true for the maximum in *N. olivacea* from April to June of the second year.

A rather restrictive interpretation of the Shannon index/evenness and the RAD analysis lead to a no observed effect level of 16 µg/L a.i..

This value is backed by the PRC and its $NOEC_{community}$ of 16 µg/L a.i..

In short, IPU treatment altered phytoplankton composition quite severely in concentration higher than 16 µg/L. Secondary effects were of great importance, even in the second year of the study. The most susceptible taxon is *Nephroselmis olivacea*, having a NOEC of about 4 µg/L even for the secondary effects in the second year. This clear class 5 effect (BROCK *et al.* 2000 in EU 2002: more than 8 week for recovery) could be used as the over-all NOEC of the phytoplankton, but one species alone is of minor ecological relevance. Ecosystem functioning is not depending on one algae alone and this one is not even the most dominant one. Other taxa of the family (Chlorophyceae) or the same size (possible limit for grazers) are not affected even at higher concentrations.

An ecological relevant over-all NOEC for the phytoplankton which is backed by several results is 16 µg/L. NOECs for the most abundant species and the $NOEC_{community}$ strongly suggest this threshold value.

4.7 Zooplankton

4.7.1 Composition of zooplankton

The zooplankton community was divided in 31 taxa: 8 taxa of Cladocera, 3 of Copepoda (including Nauplius larvae), Ostracods, 18 Rotifers and one insect larvae (Diptera), *Chaoborus crystallinus*.

Most dominant taxa are listed in Table 33.

Table 33: Dominant species in the zooplankton of the IPU study

	species	% dominance (IPU)
1	Cyclopoidae (Copepoda)	29.3
2	*Simocephalus vetulus* (Cladocera)	22.0
3	Nauplia ssp. (Copepoda)	12.0
4	*Chaoborus crystallinus* (Insecta)	6.7
5	*Mytilina mucronata* (Rotifera)	6.0
6	*Alona guttata* (Cladocera)	4.0
7	*Lecane* forma "monostyla" (Rotifera)	4.0
8	*Polyarthra vulgaris* agg. (Rotifera)	3.4
9	*Lepadella ovalis* (Rotifera)	1.9
10	*Testudinella patina* (Rotifera)	1.5

4.7.2 Abundance data

4.7.2a Total abundance

The total number of zooplankton organisms is presented in Figure 62. Deviations from the control range are not too high at any concentration of IPU. In the first year, especially in IPU5, fluctuations are evident for three months after the treatment (day 69 a.t.). In September, all enclosures reach control level again. Later on (day 97 to 139 a.t.), abundances are diminished for a second time. This pattern lasts until the middle of 2001, when IPU4 and IPU5 have higher abundances than the controls.

Generally, the data points are not in the order of the treatment intensity. Because of this fact, it was not possible to calculate a NEC. A log-linear regression is simply impossible with this structure of data. The effects seen here may stem from some secondary interaction and not from direct toxicity. As a result, a loosened treatment relation is plausible.

In contrast, calculating a NOEC using Williams' tests lead to plausible results. Williams' procedure uses "isotonized" (scaled) means for each treatment level and assumes a linear trend in the data that is related to the treatment level. It is therefore not as restrictive as a "simple" regression analysis. In the presented case, on the other hand, we have to be careful not to over-interpret the outcome of the Williams' tests, because they may "produce" a trend where there is none. Values are quite close to each other, so the scaling may lead to such a trend.

In the first year of the study, Williams' test noted a NOEC for a decline in the numbers of zooplankton of smaller than 4 µg/L IPU for five dates; two consecutive ones on two occasions, days 41/55 and 111/139, respectively. Day 7 a.t. is the fifth one which is "surrounded" by NOECs of 128 µg/L (day 3) and 16 µg/L (day 14). Keeping the notes above in mind, and looking at the curves, a no effect level of 4 µg/L is chosen for the decrease of the total zooplankton abundance.

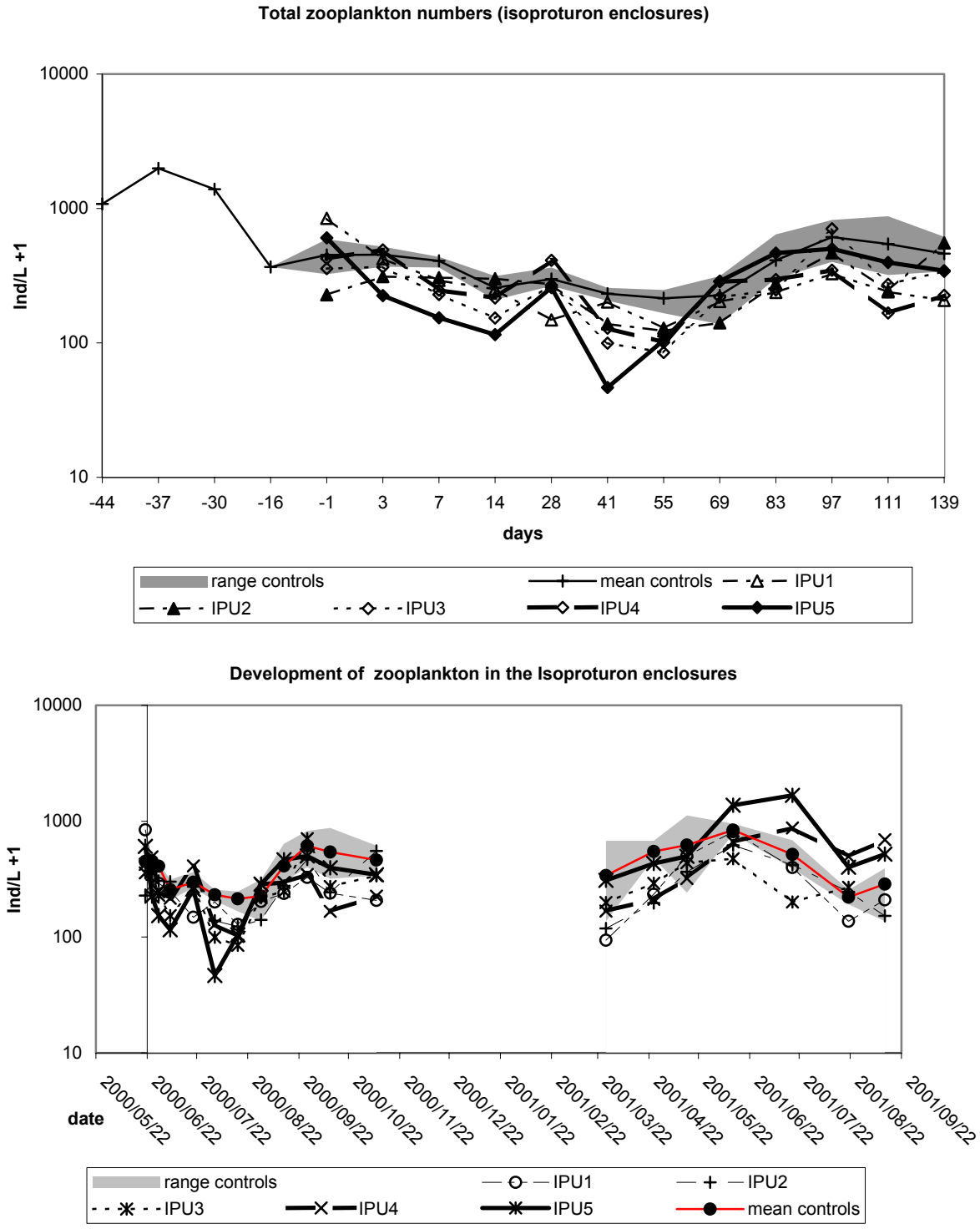


Figure 62: Zooplankton abundance in the IPU study; top: year one, bottom: both years

A clear NOEC for the increase in mid-summer 2001 cannot be noted. More zooplankton is surely present in IPU4 and IPU5, so a no effect level of 64 µg/L is sensible.

The curves presented here quite nicely resemble the ones of *Cryptomonas ssp*. (Figure 54, page 97). Even their fluctuating pattern is represented in the zooplankton. INFANTE (1973) noted that this algae, although ingestion rates are lower than for other phytoplankton species, is an important nutrition for zooplankton organisms (see also AHLGREN 1990, KIRK 1997). There might be a link via the food web: The zooplankton is affected by its food source which is

influenced by the treatment, both for the decrease and the increase[18]. The NOEC of the algae is 16 µg/L IPU. Supposing that the algae is the main nutrition in the ponds and that it cannot readily be ingested, a NOEC for the consequently secondary deviations in zooplankton might be even lower than the NOEC for the directly affected species.

4.7.2b Species richness

The number of zooplankton species found in the IPU study is depicted in Figure 63. No treatment effects were detectable in the second year of the study.

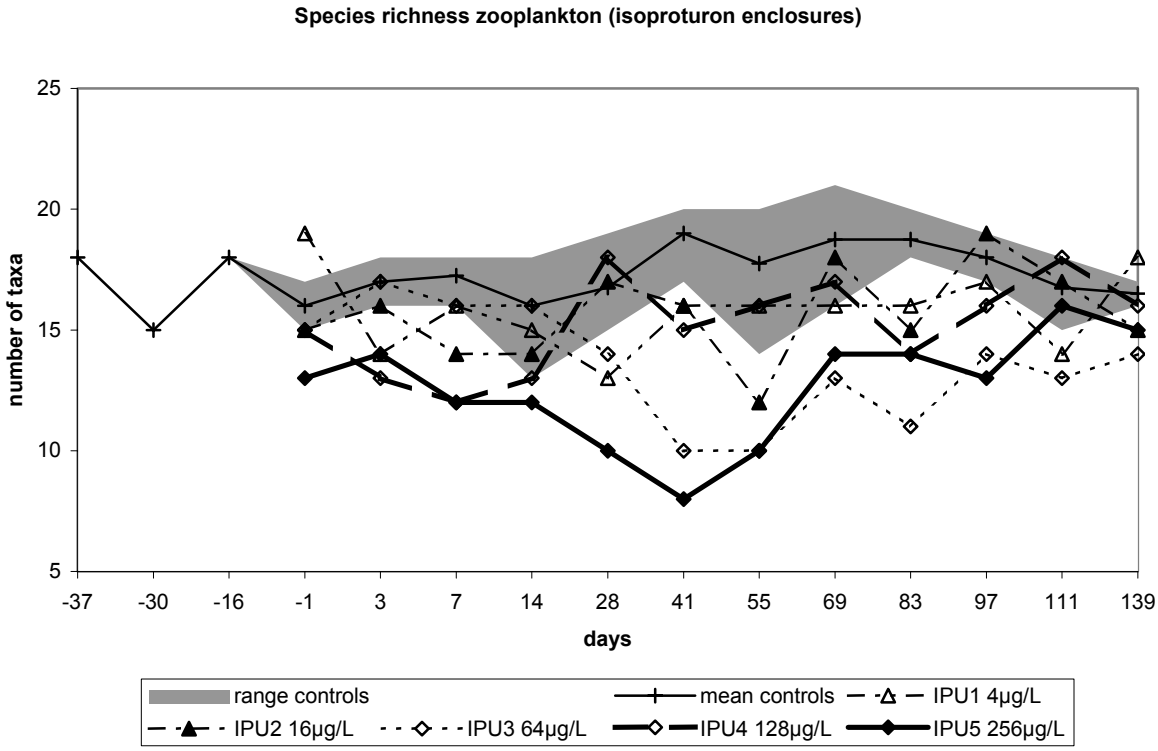


Figure 63: Species richness of the zooplankton in the IPU study

Between day 3 and 97 a.t. a decrease in IPU3 to IPU5 can be seen. IPU4 has often more taxa than IPU3, though. Here again, like in the total abundance, the curves are not clearly sorted according to the treatment level. Secondary effects like competitive exclusion (e.g. HEBERT 1982, GILBERT 1988, GILBERT 1989, SOMMER 1991, GAEDKE 1991) due to changes in food (4.6) may lead to a progression for which the amount of herbicide is not the single or exclusively important factor. NEC calculations were possible on four dates. The values are 30.6-37.2-40.8 µg/L IPU, backing the interpretation that no effects occur up to 16 µg/L a.i..

Direct toxicity may be excluded by the results of the single species tests (4.2 and 4.3). Species richness may decrease by some species falling under the detection limit of the sampling method. This can happen because of reduced reproduction under food limitation combined with pesticide treatment, especially in Rotifers (CECCHINE and SNELL 1999). This group has the highest number of taxa (4.7.1) and most taxa with a rather low steadiness in the samples (data not shown). Some of them were only found occasionally (e.g. *Euchlanis sp.*),

[18] In fact *Cr. erosa et ovata* is the most dominant algae in the test system, see Table 29 on page 92.

thus such effects are plausible. For example, on day 41 a.t. two Cladocera (*Daphnia longispina, Graptoleberis testudinaria*) were not found in IPU5, but seven Rotifers were missing (*Lecane* forma "monostyla", *Lecane* forma "diplostyla". *Lepadella ovalis* s.l., *Lepadella patella*, *Mytlina mucronata*, *Testudinella patina*, *Trichotria pocillum*).

Complete recovery in species richness can be noted on day 111 a.t..

4.7.2c Chaoborus crystallinus

The dipteran larvae were not susceptible to the herbicide at all. No secondary effects could be found. The total abundance of the larvae's prey varied to some extend over the period of the study (4.7.2a). In any case, this variation was not strong enough to result in detectable changes in the abundances of the predator. Differences in the top-down control of the system like the ones seen in the CYP study were not present here.

4.7.2d Nauplii

The numbers of Nauplius larvae in the system decrease with time in all enclosures (Figure 64). Except for day 14 a.t., all enclosures treated with more than 16 µg/L IPU are below the control range up to day 55 a.t.. Values are quite nicely related to the treatment.

From day 83 to the end of the first year (day 139), the smaller abundances in the influenced enclosures do not relate to the amounts of IPU applied anymore. IPU3 has least counts, the others are more or less in the same range. The values in all enclosures are just slightly above the number of individuals per liter that are needed to be worth being explored statistically (10/L, MAISE 2002). Nevertheless, secondary effects may lead to this altered behavior.

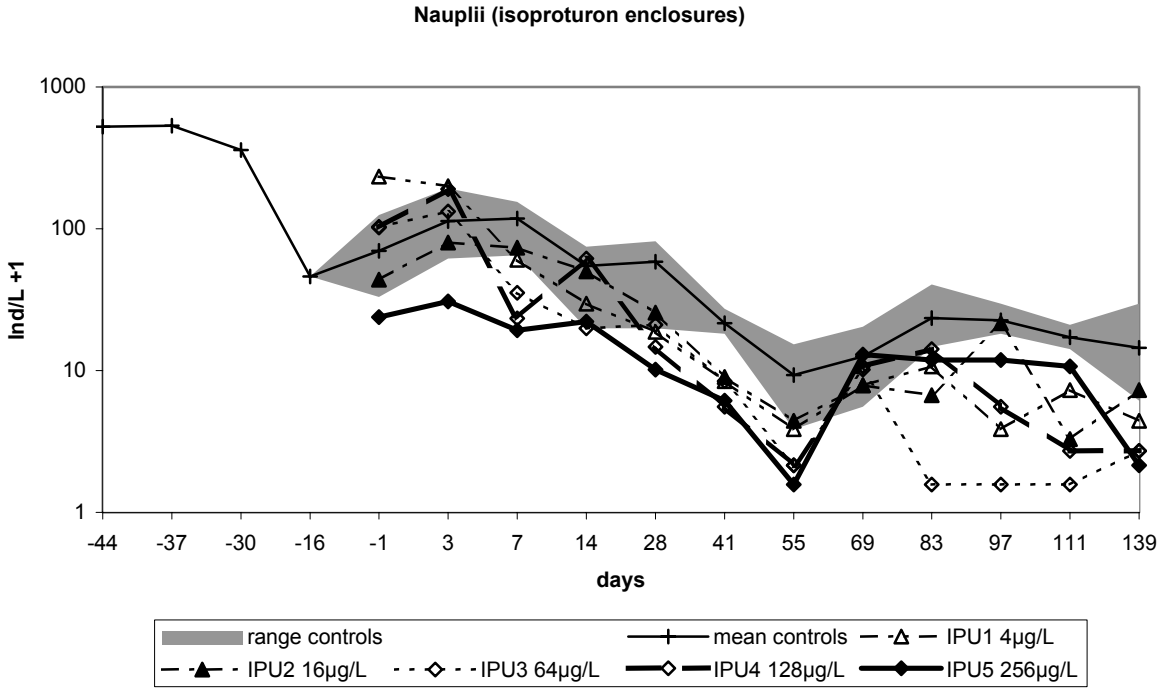


Figure 64: Development of naulius larvae in the IPU study

No effects were seen in the second year. Recovery is noted for spring of the second year of the study.

The NOEC is very low, less than 4 µg/L. Calculations were significant ($p<0.05$) for this value seven times, twice in groups of three consecutive dates. The only NEC values that are sensibly low (within the treatment range) are the ones defined by the intersection of the log-linear regression with the upper 95% confidence intervals (i.e. the lower NEC values). Their mean is 92.1 µg/L, that means over twenty times higher than the NOEC. This relationship can be explained by the low slope of the regression curves on data points that do not show big differences.

As aforementioned the numbers are quite low to the end of the year and the reduction is not too great over the whole time, so the ecological relevance of the high sensitivity is at least questionable. Care has to be taken because there is only one (or two) generation(s) of the Copepods per year (SOMMER 1994). In the following the juveniles and adults are investigated for treatment effects.

4.7.2e Cyclopoida

The development of the Cyclopoida in the IPU enclosures in presented in Figure 65. This taxon is the most dominant one (Table 33), so it is not surprising that is shows a progression resembling the total abundances (Figure 62).

Differences can be seen right after the application, when the Copepods show no reaction at all (whereas the total zooplankton abundance declines). Therefore it can be assumed that the Cyclopoids are not sensitive towards IPU at all. Deviations are supposed to be of secondary nature.

From July on (day 28 a.t.), Copepods enter the fluctuating pattern seen so often in the data of this study. Again, this may be due to the link via the food web.

From day 83 a.t. (September 2000) until the end of the study, numbers of Copepods are low in IPU3. One the one hand, this explains the low numbers of Nauplii in this pond (Figure 64): fewer adults produce less offspring. On the other hand less offspring leads to lower numbers in the next generation (data of adults in 2001). This indeed can bee seen here. No other effects of the reduced numbers in larvae can be seen in the Copepodit and adult stages. IPU4 or IPU2 do not show these or similar effects. Thus, the outcome of the competition must have been unfavorable for the Cyclopoids solely in IPU3 (see also 4.7.2g).

IPU5 sees an increase in the abundance of the Cyclopoids between autumn 2000 and mid-summer 2001. It is in line with the controls again from May 2001 till the end of the sampling period. The other enclosures show only minor deviations from the control range.

Explanation may be given by the IPU residues that are still present in the system in that period of time. The heightened abundance stops when enough IPU is removed from the water column (see also 4.1). Before that time, the Cyclopoids seem to be the better competitors. Keeping the long generation time in mind (cf. the last paragraph of 4.7.2d), mortality must be reduced in order to reach higher numbers. This is only possible due to competition for food, because the top predator, *Ch. crystallinus*, is not affected by the treatment (4.7.2c). Copepods are selective feeders that show chemotaxis (SOMMER 1994), so advantages (finding the most nutritious algae, avoiding dead ones etc.) may well exist for them.

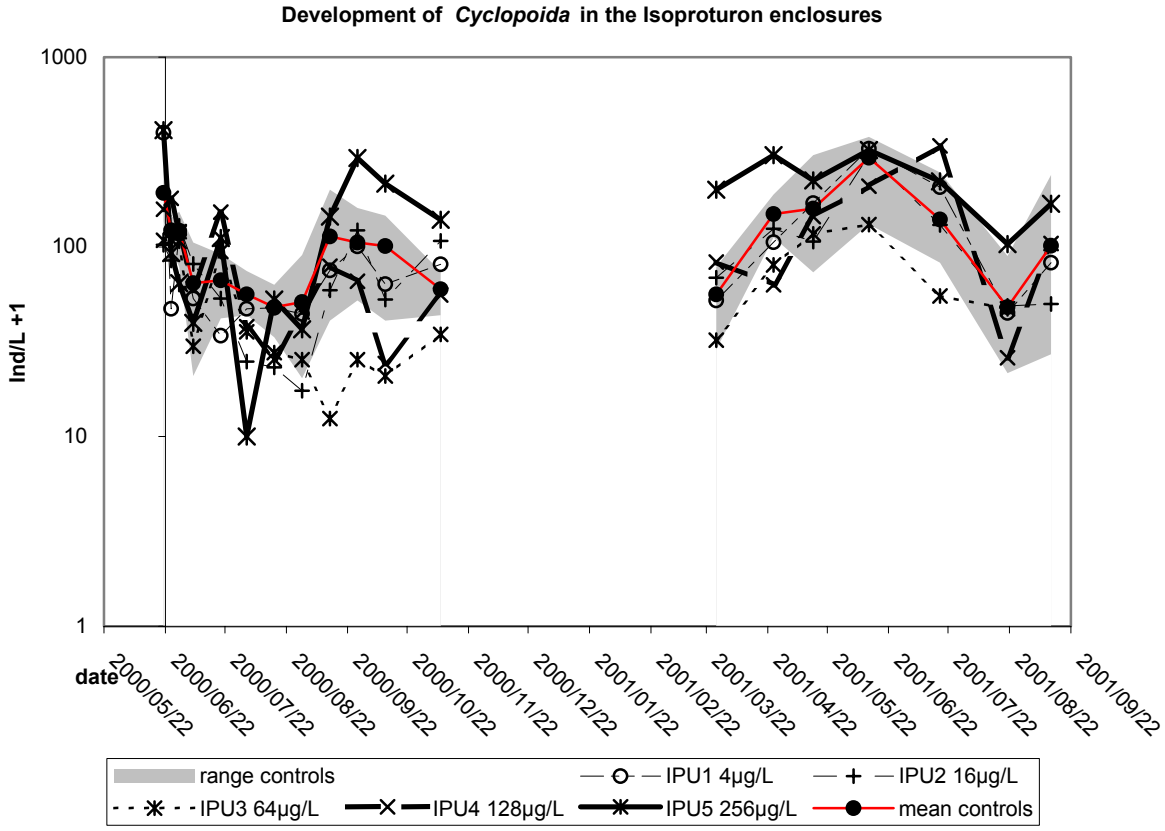


Figure 65: Development of the Cyclopoida in the IPU enclosures

A different explanation may be due to the reduced macrophytes in the system (4.5). The artificial substrates may attract more animals if there are less submerged structures present. The low numbers in IPU4, that also had less macrophytes, is opposed to this interpretation.

A NOEC of 128 µg/L IPU was calculated for the increase in autumn to spring. NEC is 86.5 µg/L (lower value, n=3), that means higher than the concentration in IPU3.

Concisely, merely secondary effects of IPU on the Cyclopoids could be seen at concentrations ≤ 128 µg/L a.i..

4.7.2f Eudiaptomus gracilis

Counts of this calanoid species were fairly low, most of the time below 10/L (3.4/L in the IPU enclosures and 4.2 in the controls). MAISE 2002 set this value as a minimum for statistical evaluation for studies with few replicates.

No treatment related deviations could be seen in the progressions of the abundances. NOEC and NEC calculation lead to inconsistent results. Whether or not this species is affected by the herbicide treatment cannot be clarified with this study. Direct toxic action can be excluded by the results of the single species test (4.2).

4.7.2g *Simocephalus vetulus*

This species is a large Cladoceran and one of the most important grazers in the system (see the results of the CYP study). Its numbers are depicted in Figure 66. Dominance analysis mentioned it on second position (Table 33). Changes here may well be of great importance even for other species due to competition alterations (NORBERG 2000).

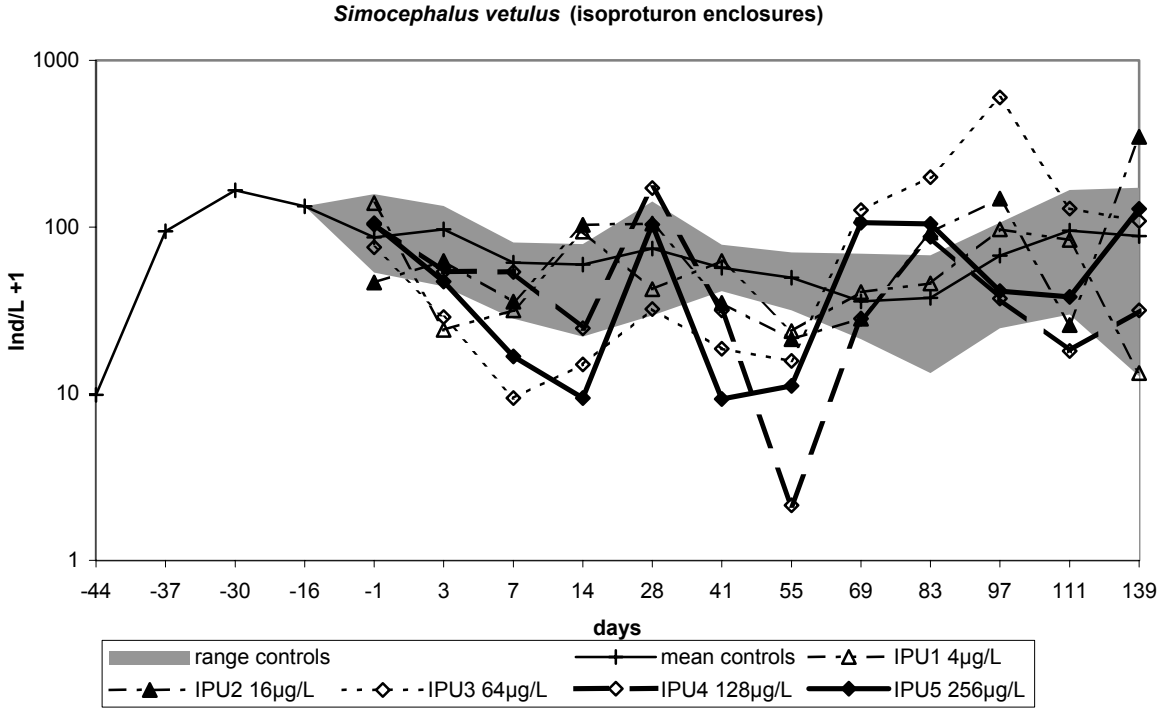


Figure 66: Development of Simocephalus vetulus in the IPU study

In Figure 66 there is a decrease in IPU5 and IPU3 up to day 28 a.t., when numbers start fluctuating. The abundance in IPU5 is lower than in the controls on days 41 and 55 and higher on days 63 and 83. Counts in IPU4 are smaller than in the controls on days 41, 55, and 111 a.t. and higher on days 28 and 83. IPU3 has fewer *S. vetulus* between day 3 and 55 a.t. (except day 28 a.t.) and the highest numbers in the whole study between days 69 to 97 a.t.. IPU2 has a wavy curve with some samples slightly out of the control range (days 14, 41, 55, and 83 to 139 a.t.). As a result, visible effects start in IPU2.

In 2001, no effects were observed. Curves are noisy, but stay within the control range most of the time. Deviations could not be addressed to the treatment or any secondary reason.

The pattern found in the first year alludes to the ones of the total zooplankton abundance (Figure 62) and the *Cryptomonas ssp.* (Figure 54). An interaction between the grazer and the algae can explain the pattern. Less food, or food of poor quality (due to reduced photosynthesis) may lead to a higher mortality in the filter feeding Cladoceran. Thus, less grazing on the algae takes place and they can reach higher numbers again. Consequently, grazers may increase again and the cycle begins once more (predator-prey interaction with negative feedback, SOMMER 1994).

The high abundance in IPU3 matches with the low in the Cyclopoids (Figure 65). A maximum in algae proceeded this development (Figure 51), enabling zooplankton growth. The

Cladoceran seems to out-competition the Copepoda under the special environmental conditions[19] of this enclosure. IPU treatment lead to a shift in the relation in numbers between the two taxa.

No NEC could be calculated, NOEC is 4 µg/L between days 3 to 14 a.t.. This finding is corroborated by the curves that leave the control range in or after this time slot. A minor toxic action of IPU on the taxon can be excluded by the biomonitoring and the single species tests (4.2 and 4.3).

4.7.2h Chydorus sphaericus

This species is a small Cladoceran that is associated to submerged structures in the littoral of small water bodies (STREBLE and KRAUTER 1988). In the IPU study, it was present in low numbers in the first year but it became more and more abundant between autumn 2000 and mid-summer 2001 in the controls (Figure 67).

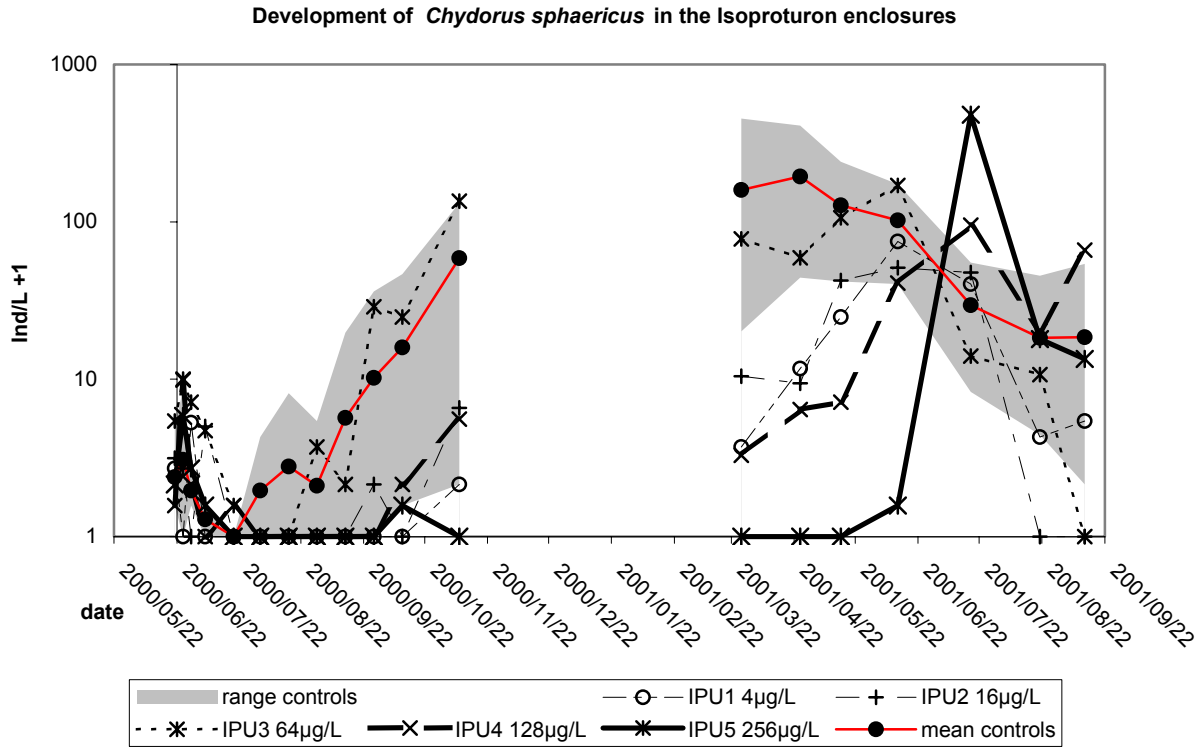


Figure 67: Chydorus sphaericus in the IPU ponds

[19] Amount of the herbicide, macrophytes cover, water quality etc.. Especially the macrophytes may serve as a reason. They are not affected in this treatment level (4.5). *S. vetulus* is associated to plants (FLÖßNER 1972). Ecosystem structuring elements like plants strongly influence zooplankton community structure (e.g. COTTONIE *et al.* 2001). Since there are less macrophytes in IPU4 and IPU5 as well as less herbicide in IPU1 and IPU2, IPU3 may show a special development by having competition for food altered by the herbicide action on algae already ($NOEC_{community}$ 16 µg/L) and simultaneously it is showing no effect on the macrophytes (IPU3 is the NOEC). Also note the generation time: approx. three weeks (FLÖßNER 1972) for *S. vetulus* and about one year for the Cyclopoids (SOMMER 1994). *S. vetulus* is therefore capable of reacting more quickly to changes in the system and can thus out-compete other, "slower" species (taxa with a smaller numerical reaction, SOMMER 1994).

Due to the low numbers at the time of the treatment, no statistical evaluation was possible in the first year. Curves indicate a decrease with treatment, though. There were no individuals found at all between days 41 to 83 a.t. in all treated enclosures except IPU3. In IPU5, no individuals at all were found until June, 2001. Whether toxic action or secondary reasons are the cause for this development cannot be clarified.

In spring 2001, again excluding IPU3, abundances are quite nicely related to the treatment level: the more herbicide, the less of these water flea. IPU1 and IPU2 have almost the same values. For this period of time (March to May), the NOEC was <4 µg/L IPU. NEC (n=3) is 59.1-72.9-(485.3) µg/L IPU, that means considerably higher. Delayed effects via the food web may be the reason for this development. Changes in food abundance and/or quality as well as competition effects could be inducing such a decline. Toxic effects should not be a reason; otherwise the values of IPU3, right within the control range, would be implausible. *Ch. sphaericus* can use periphyton as food source as well (LAWA 1996), that the Cladoceran scrapes off the substrate. The changes in silicate, that are not matched by the development in planctonic diatoms (see 4.4.7b and 4.6.2e), indicate a decrease in periphyton (lowest concentration with no effect around 16 µg/L IPU). Changes in periphyton could be seen by ESER (2001) at concentrations of 25 µg/L IPU and higher. PÉRÈS *et al.* (1996) found a reduced diatom abundance in periphyton even at 5 µg/L IPU. An impact on the grazer may therefore be conceivable. Another reason may be an impact of IPU on bacteria. These organisms serve as food for *Chydorus*, because the mesh aperture of its filter apparatus is about 0.2-0.3 µm (SOMMER 1991, 1994). Effects on bacteria, especially due to differences in the amounts of detritus (i.e. secondary impact!), cannot be excluded although they were not investigated in this study.

From June 2001 to the end of the study, IPU1-3 exhibit a decline, whereas IPU4 and 5 are above the control mean. This may be due to changes in the whole system by the time no more IPU is in the system. Macrophytes start growing again. Only the most vigorous ones may have survived the treatment. As a result, they can grow better than the unaffected ones even when conditions get worse in autumn. In the lowly treated pond, the plants may be weaker because they were not able to produce enough reserve material due to the reduced photosynthesis under IPU influence. The microhabitat structure consequently changes to some extend. *Ch. sphaericus* can take advantage in the highly treated ponds (food and spatial niche) and is adversely affected in the lower IPU levels.

All the effects seen in 2001 are almost certainly secondary ones. No direct link to any other of the parameters investigated was found, thus a combination led to the development (IPU concentrations, macrophytes, competition etc.). It is important to keep in mind that numbers in IPU1, 2, and 4 already began to rise in autumn, but not in IPU5. Generation time for clacocera is about 2-3 weeks (FLÖßNER 1972), so the increase is rather slow. Interactions in the food web may delay the development together with the lower temperatures in autumn and spring.

IPU3 has to be dealt with separately. It does not fit in the explanations given above, because it is in the control range most of the time. Three reason can be given for this:

1. This enclosure is received the highest dose of IPU without affecting the macrophytes (4.5);
2. Numbers of Cyclopoids and their larvae are lower in IPU3 than in the other ponds (4.7.2e);
3. Numbers of *S. vetulus* are higher than in the other ponds in autumn 2000 (4.7.2g).

At least the combination of these reasons apparently favored *Ch. sphaericus*. Which one is the most important rationale cannot be derived from the data of this study.

4.7.2i Rotifera

The Rotifers in the IPU study are presented in Figure 68. Quite notable differences exist even before the application. Toxic effects may be hinted at in the beginning but cannot be considered too certain from looking at the curves alone. A comprehensible treatment relation could not be found in the abundance data. In the first year, IPU5 has low abundance counts until August, whereas later on they can be found around the ones of the controls till the end of the year. IPU4 does not deviate from the controls very much at any time in this year. IPU3 and IPU1 have lower abundances beginning on day 41 a.t.. IPU3 is affected more severely (excluding day 111 a.t.). The deviations from the control range may be of secondary nature again. IPU2 does not leave the control range substantially during the whole sampling period.

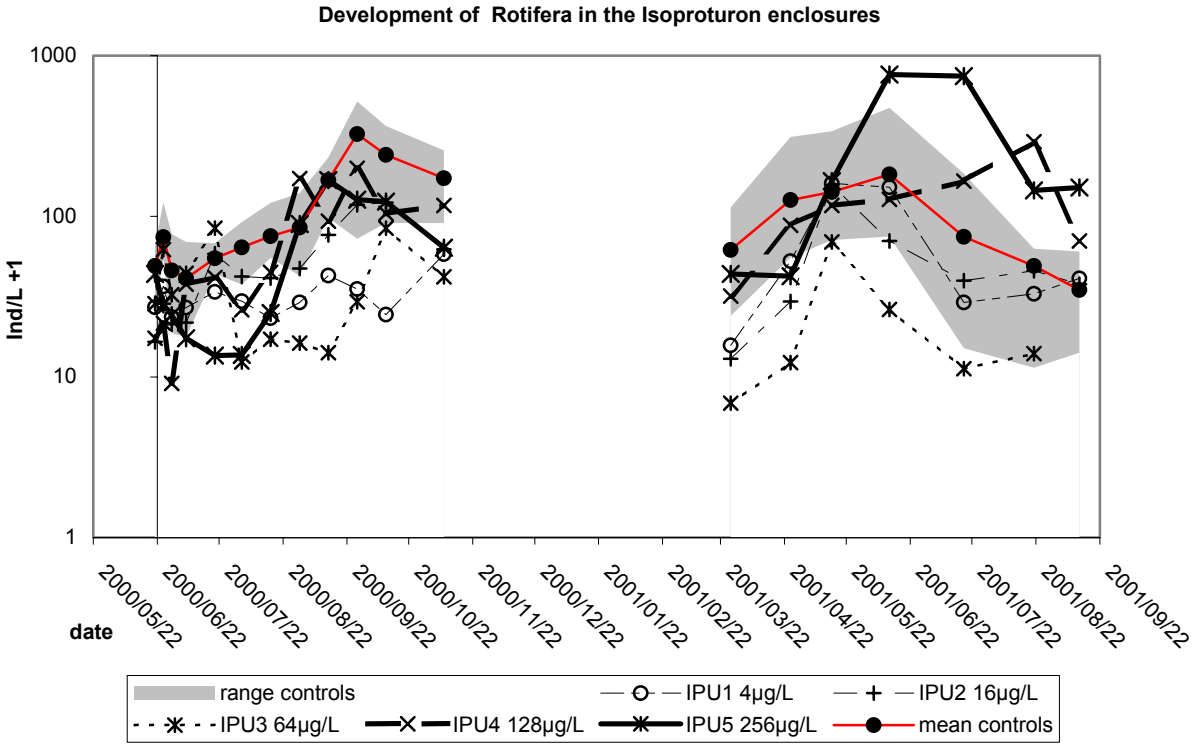


Figure 68: Development of the Rotifera in the IPU study

The low numbers in IPU1 cannot be explained readily; maybe it is "just" a special feature of the enclosure. The impact in IPU3 may be because of competition reasons. This enclosure had a special development in the time slot in question in many other taxa (*Nauplii, Cyclopoida, S. vetulus, Ch. sphaericus*). For this reason, a peculiar development in Rotifers is possible, too.

The development in 2001 in IPU3 (low abundance again) can be explained in the same way as above. All the other enclosures are more or less in line with the controls until June. From this point of time, IPU4 and IPU5 have higher numbers of Rotifers. The macrophytes which them start to grow again may serve as an explanation, because they alter the ecosystem quite substantially (e.g. COTTONIE 2001).

A NEC could not be calculated. Williams' tests found a NOEC of 4 µg/L (day 3-41 a.t. and day 97-298 a.t.). The low value may be due to the special development in IPU1 and can therefore not be generalized without further thought. The increase at the end of 2001 was significant in concentrations higher than 64 µg/L IPU (day 391-447).

Giving a concentration of IPU that has no impact on Rotifers is difficult. Complex interactions seem to cause the effects and IPU1 has a special development in the presented study that could not be explained. A no effect level of 16 µg/L may be reasonable, because secondary effects in this concentration were of minor importance for most tested endpoints.

4.7.3 Community analysis

4.7.3a Shannon index and evenness

The mean value of the evenness in the IPU enclosures is 0.64, and 0.66 for the controls. Shannon index has a mean of 1.72 in the treated ponds and 1.89 in the controls. Again, the development is nearly identical and the evenness is better when comparing the data (see CYP part). Data are graphically displayed in Figure 69.

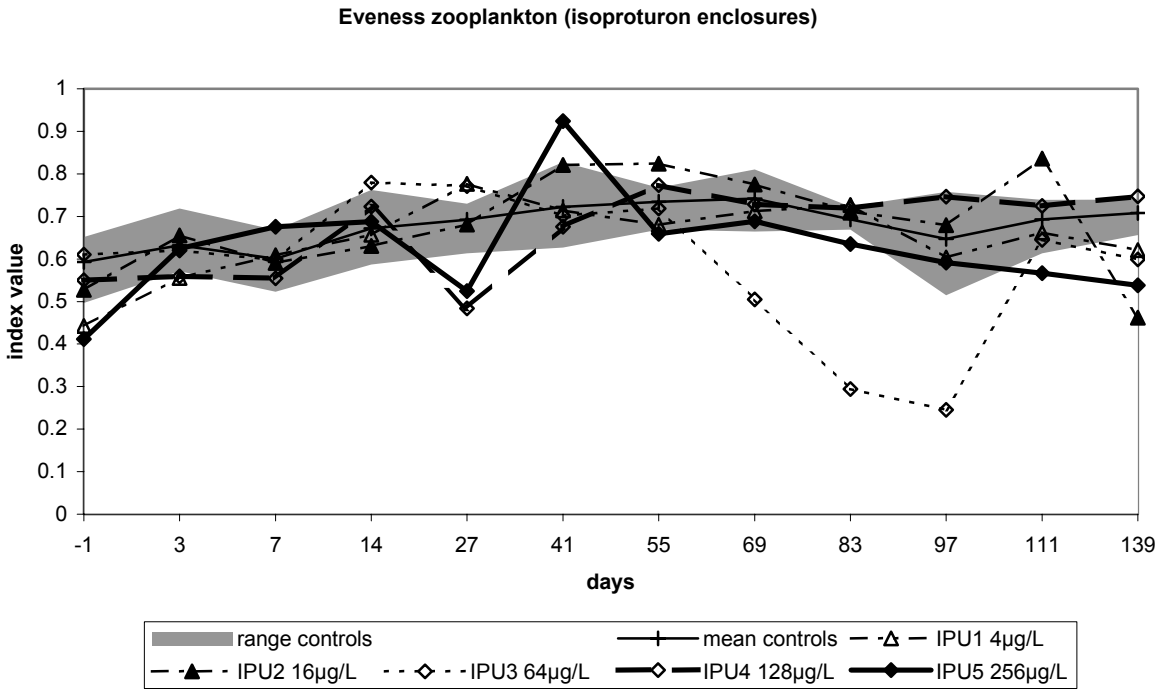


Figure 69: Evenness of zooplankton data (IPU study)

Effects are restricted to concentrations higher than IPU2. The fluctuating pattern of *S. vetulus* and the Cyclopoida in IPU4 and IPU5 is reflected here between day 14 a.t. and 55 a.t.. IPU3 has a distinct decrease between day 69 and 97 a.t.. This is due to the changes in Cyclopoida, Nauplii, Rotifers, and *S. vetulus*. IPU2 exhibits only minor deflections. NEC

values are presented in Table 34. In this case, these diversity indices are not too sensitive compared to other endpoints.

Table 34: NEC values of the evenness and Shannon index of zooplankton (IPU study)

NEC [µg/L]	Evenness	N	Shannon	N
lower	63.98	4	94.60	3
middle	128.09	4	114.31	3
upper	392.84	4	186.03	3

Even the lower values are above IPU3, but the effects in this enclosure are pretty pronounced. A log-linear regression is impossible with the concentration in the middle as it has the biggest deviations. Effects cannot be estimated correctly by this type of analysis. A no effect level of IPU2, 16 µg/L is chosen for this reason.

4.7.3b RAD index

The RAD index is presented in Figure 70. For IPU5 it is out of the control range for the whole study except for day 355 and 447 a.t.. For IPU4 it is rather wavy and slightly in line with or over the controls. Differences in abundances of many taxa were found at these treatment levels, so the influence of IPU is evident. Because macrophytes are affected by the treatment at levels above 64 µg/L, the microhabitat structure of the ecosystem is changed and this leads to changes in the zooplankton (see also 4.5).

IPU1 and IPU2 have only temporarily increased data in autumn 2000 (days 83-139 a.t.). These can be interpreted as seasonal effects altering the zooplankton in a different way in each enclosure. Effects of the herbicide are of minor importance at these concentrations (see data above).

IPU3 shows the highest values in autumn 2000 (days 69-139 a.t.). This finding can be explained by the secondary changes in Cyclopoida (4.7.2e), Nauplii (4.7.2d), Rotifers (4.7.2i), and *S. vetulus* (4.7.2g). The development of these taxa in IPU3 is different from the one in the other enclosures, so that the high RAD is readily understandable. The high value of IPU3 on day 447 surely is an outlier.

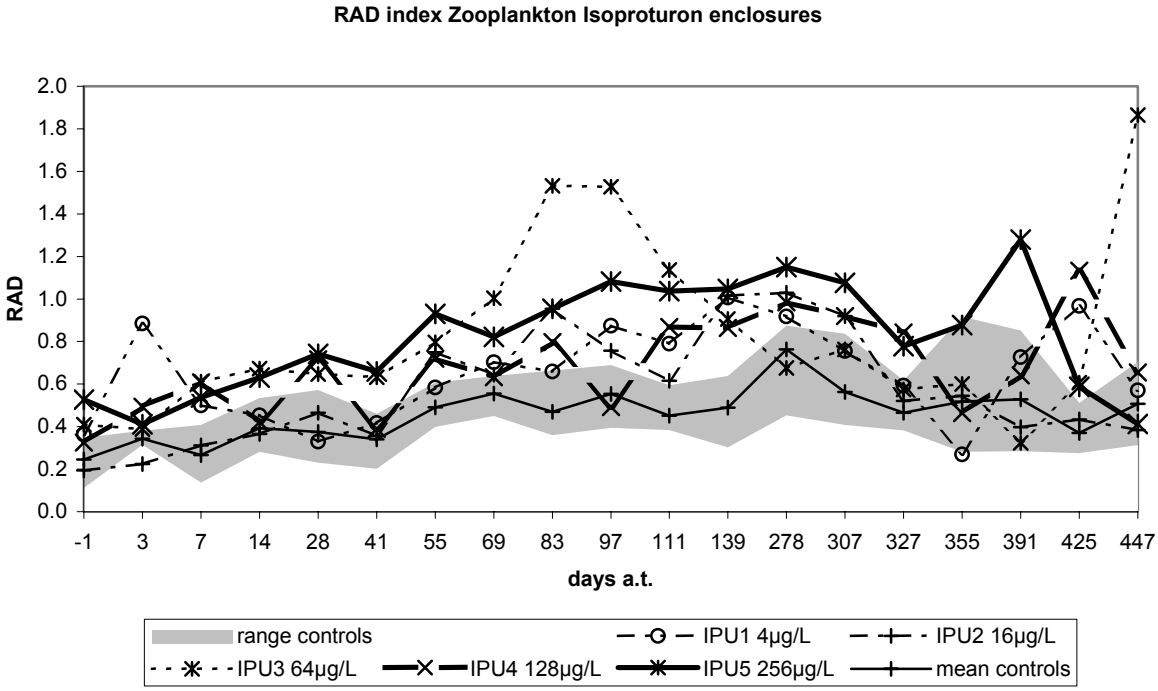


Figure 70: Development of the RAD index (IPU study)

NEC calculations were only possible in 2000. Values are 9.2-13.2-20.9 µg/L IPU. Consequently, no effects are expected at concentrations up to 16 µg/L (IPU2).

4.7.3c PRC analysis

Multivariate analysis data is depicted in Figure 71. IPU5 deviates notably between day 14 and 55 a.t.. IPU4 is fairly wavy again. The deviations of IPU3 between day 69 and 139 are reflected here, too. The other ponds step by step diverge from the controls (x-axis).

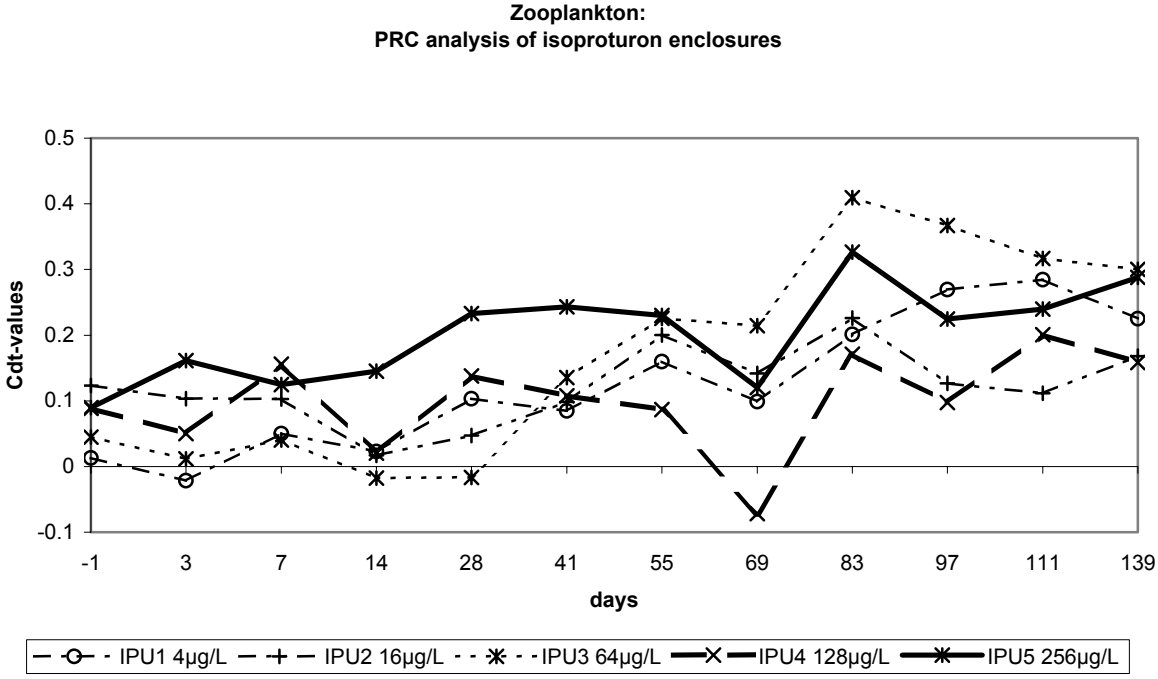


Figure 71: PRC analysis of the zooplankton in the IPU study

This analysis is significant, p=0.015. It explains 40.4% of the variances by the treatment of which 32.1% are displayed. 39.3% of the variations are explained by the sampling day. Species scores are listed in Table 35.

Treatment effects are visible but not too well related to the amount of IPU used. As already said in above analysis, impacts are of secondary nature and thus a somewhat changed relation is possible. Recovery could not be demonstrated.

Table 35: Relevant zooplankton taxa in the PRC of the IPU data

Taxon	score
Lepadella ovalis s.l. (Rotifera)	-0.61
Mytilina mucronata (Rotifera)	-0.60

The two species found important have a rather low abundance: *Mytilina* has 7.8/L as the mean (5.2/L up to d83 in the IPU enclosures, and 7.3/L in the controls). *Lepadella* abundance was even lower. Treatment effects were thus not detectable because of first, the very high fluctuations which are normal for these low abundances, and second, the low numbers themselves which cannot be dealt with statistically.

$NOEC_{community}$ is 64 µg/L IPU; NEC is in this range: 54.5-94.4-121.0 µg/L (n=3); thus always below IPU4. The effects in IPU3 are therefore neglected by the analysis.

Again, the PRC is not the best tools when investigating secondary effects of pesticide actions. Species more susceptible to changes under herbicide influence were not detected.

4.7.4 Overview of treatment effects of IPU on the zooplankton

A summary of several NOECs of zooplankton data is given in Table 36. Note that all the effects found were secondary ones. Toxic action of IPU on zooplankton was never seen here and is corroborated by the findings of biomonitoring and single species tests (4.2 and 4.3). Being a photosystem II inhibitor IPU is not expected to adversely influence animals directly. Distinct treatment effects occurred at concentration higher than 4 µg/L IPU.

Table 36: NOEC data for some zooplankton taxa and endpoints of the IPU study

	NOEC [µg/L]	remark
Chaoborus crystallinus (Insecta)	no effects at all	not susceptible
Chydorus sphaericus (Cladocera)	4 µg/L	only second year with clear effects, IPU3 with special development
Cladocera	16µg/L (days 3-14 a.t.)	decrease
Cyclopoida (Copepoda)	≤ 128 µg/L (increase in autumn)	not sensitive, secondarily affected
Nauplii (Copepoda)	<4 µg/L	minor decreases, only in the first year, esp. IPU3
Rotifers	16 µg	secondary effects, IPU1 and 3 with special development
Simocephalus vetulus (Cladocera)	4 µg/L	secondary influence on the whole plankton community
total abundance	4 µg/L	decline in the first year, increase in the second year beginning in mid-summer
$NOEC_{community}$	64 µg/L	PRC is rather insensitive towards secondary effects

Generally, complex interaction between algae and grazers has previously been found to influence biodiversity as well as ecosystem functioning (NORBERG 2000). Food and grazers are linked to each other by many feedback loops (a review is given by TILZER 2000). Secondary and even tertiary effects can thus be expected in zooplankton, because the phytoplankton is altered by the treatment. Effects in grazers may also influence phytoplankton again.

Interactions were seen in the total abundance. Zooplankton numbers were closely linked to the abundance in *Cryptomonas ssp.*. Decreases in the algae lead to decreases in the zooplankton. The feedback was very tight, so reduced grazing pressure enabled the algae to grow again. Zooplankton thus increased secondarily. An oscillating pattern in both parameters was the result in the treated enclosures. KERFOOT (1989) noted only minor changes in algae due to altered zooplankton densities in summer, because the algae then are under resource limitation. As presented here, abiotic parameters were altered by the treatment (4.4). The algae were provided with more mineral nutrients while the effects of the herbicide get less with time. As a result, the oscillations were facilitated to build up each other. This effect was resilienced in autumn, when major changes in the ecosystem, preparing itself for winter, took place. Secondary interactions were not seen in IPU1 only, i.e. 4 µg/L IPU. The balance between algae and zooplankton was rather delicate.

In the following year, effects on macrophytes as well as the complete degradation of IPU in the water column enabled zooplankton densities in the highly treated ponds to exceed control level.

Species richness was also negatively affected to some extend at concentrations above 16 µg/L in the first year. Rotifers have the biggest impact on this development.

Chaoborus crystallinus abundance was not changed by the treatment. Deviations in prey had no influence in its numbers.

In Copepods, Nauplius larvae were very susceptible to the treatment with a NOEC of less than 4 µg/L IPU. Decreases were not too bad, though. No effects were seen in the second year. In the adults, the NOEC is ≤ 128 µg/L. In the highly treated ponds the number of adults showed an oscillating pattern shortly after the application of the herbicide and an increase in late 2001. No effects of the lowered abundance of the larvae occurred. Consequently, the low NOEC in larvae is of minor ecological importance. IPU3 showed a special development (lower abndance) in larvae and adults which can be explained by the outcome of competition under the conditions of living in this treatment level.

The species most directly linked to the algae (again the *Cryptomonas ssp.*) is *Simocephalus vetulus*. This big Cladoceran is an effective grazer (see CYP part) and clearly controlled bottom-up by the algae. Its development is similar to the total abundance in zooplankton. Deviations in this grazer induced changes in other plankton taxa as well (see especially the abundances in Cyclopoida and *Chydorus sphaericus* in IPU3). *S. vetulus* is very sensitive with a NOEC of 4 µg/L IPU.

Chydorus sphaericus is negatively affected in the second year. Abundances in the first year were too small to deduce statistically significant effects. Negative effects seemed possible, though. NOEC could be about 4 µg/L. The development in IPU3 allows conclusions about competitive interactions (see above: IPU3 abundances was in the control range all the time and mostly higher than in the rest of the treated enclosures).

Community analyses were not as sensitive as investigating changes in the abundances. RAD and evenness/Shannon index had a no observed effect level of 16 µg/L. PRC was not able to detect the most susceptible species. The $NOEC_{community}$ is thus quite high, 64 µg/L.

4.8 Summary of the IPU study effects

IPU was rather resistant in the water column. It took about one year until the highest treatment level was decontaminated. Half-life time was about 16 days in the water column.

Zooplankton species used in the single species tests and the biomonitoring were completely insensitive towards the herbicide even at concentrations of 1000 µg/L a.i..

The herbicide had distinct influences on the model ecosystem. Effects in all water quality parameters were apparent at concentrations of 16 µg/L and more (NOEC=4 µg/L); water chemistry showed deviations beginning at 64 µg/L IPU (NOEL=16 µg/L). A DO-pH-alkalinity-conductivity syndrome could be demonstrated.

Macrophyte cover was reduced at the two highest levels. This is a major change in the ecosystem structure that was reflected in all other parameters investigated.

Phytoplankton was sensitive towards the treatment (over-all NOEC 16 µg/L). At least two algae, *Chroomonas acuta* (NOEC 16 µg/L) and *Nephroselmis olivacea* (NOEC ≤4 µg/L) were considerably cut down in numbers. *Cryptomoans ssp*. were sensitive, too (NOEC=16 µg/L). Secondary effects led to an oscillating pattern in the development. Zooplankton, especially *Simocephalus vetulus* and the Cyclopoids, reacted to this pattern. In Figure 72 the interaction in

the controls between *Simocephalus vetulus* and phytoplankton is presented. Changes are rather small, but an increase in the grazer lead to decreases in the algae.

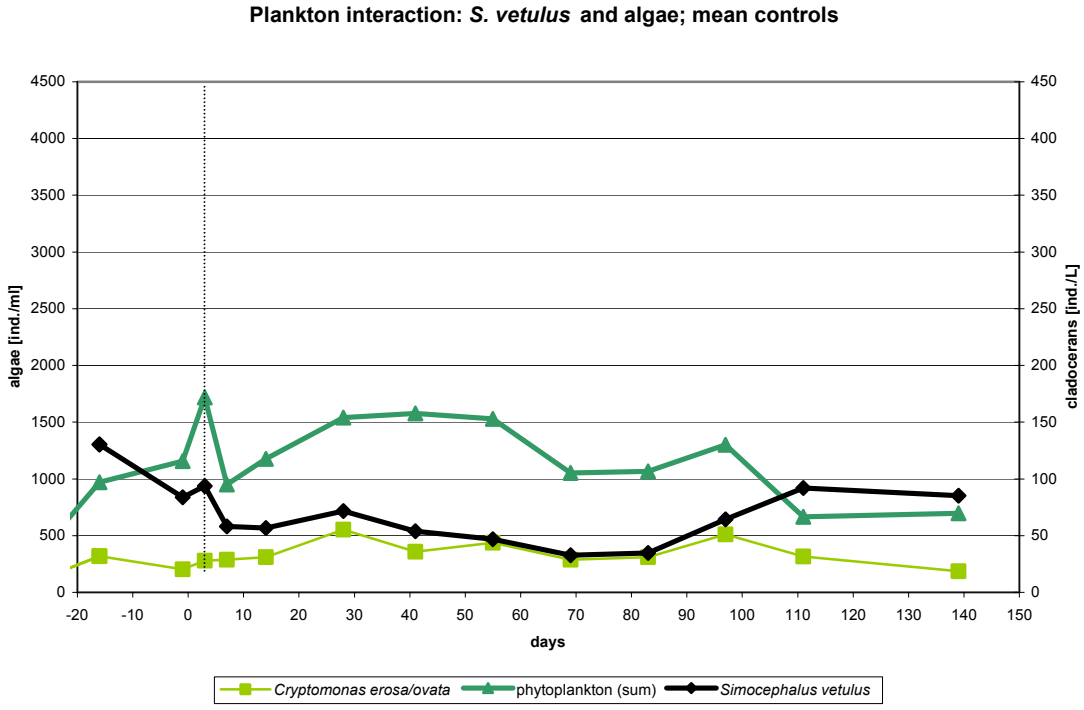


Figure 72: Interaction of plankton in the IPU study, control data

In Figure 73 the same interaction is shown for IPU5. Here the algae decline after the application (days 0-7 a.t.). Simultaneously, the grazers get fewer. After the first week, their abundance must have fallen under a threshold for the algae to be released from top-down control. They reached a maximum on day 14 a.t.. About two weeks later, after about one generation period of the Cladoceran, the grazers increased in counts. More food may have led to more offspring. Then again algae were decreasing. This pattern was continued up to day 97 a.t., in September. Lower treated enclosures showed a similar development with lower amplitudes and less speed (thus less maximums).

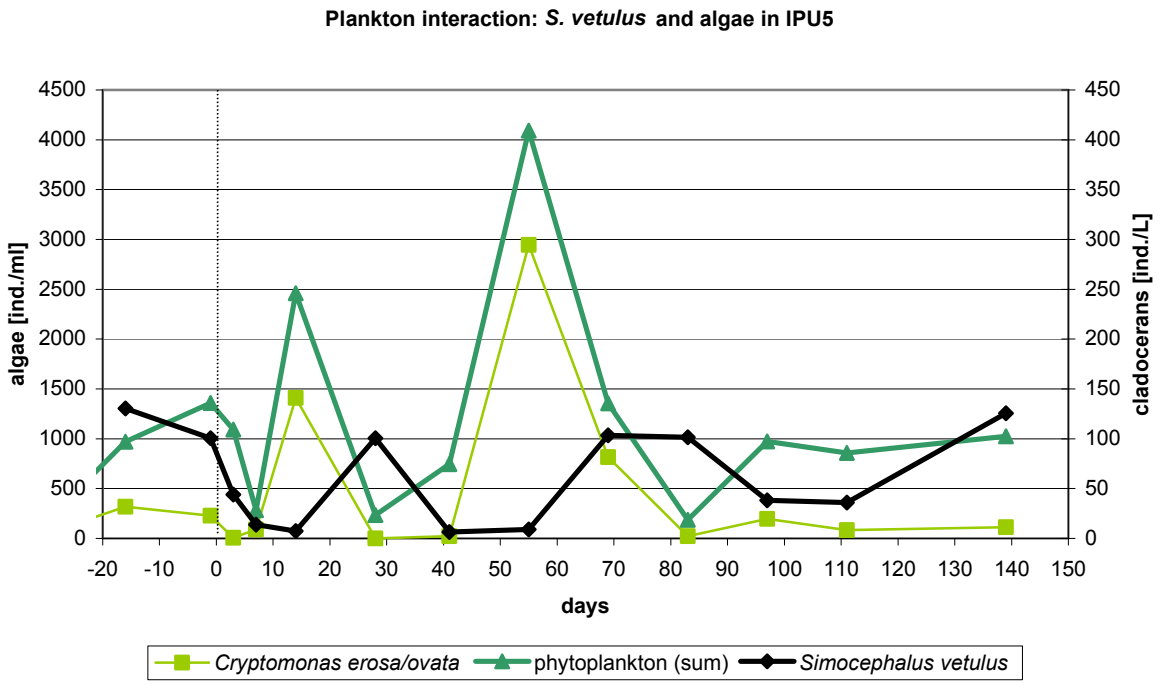


Figure 73: Interaction of plankton in the IPU study, data of enclosure IPU5 (256 µg/L IPU)

These oscillations triggered the same pattern in other zooplankton taxa (e.g. Cyclopoids). IPU3 with its "middle" position with an already altered phytoplankton structure ($NOEC_{community}$= 16 µg/L) and unaffected macrophytes shows special interaction results: The plant associated Cladoceran *Chydorus sphaericus* is favored strongly while the Cyclopoids are decreased. -

Direct toxicity of IPU on the zooplankton was never observed. The secondary effects had a NOEC of 4 µg/L. This is less than for the algae (16 µg/L). The secondary effect is controlled bottom-up and may thus be more sensitive because the grazers' mortality *and* reproduction are affected:

The quality of the food may well deteriorate because of less photosynthesis products in the algae without changing the abundances of the algae (therefore not detected here). The amount of primary production is surely changed (see the lowered values of the oxygen content). DORIGO and LEBOULANGER 2001 noted an EC_{50} for photosynthesis (fluorescence measures) of 14 µg/L IPU (lowest value) in periphyton. ESER 2001 found the phytoplankton about half as sensitive to IPU, NOECs of 20 µg/L and 36 µg/L, respectively. Consequently, differences in the food quality due to IPU treatment at no-lethal concentrations (for the algae) seems viable. RIOBOO *et al.* 2002 found changes in cell dry weight (higher), C/N ratios (lower) and protein content (higher) in *Chlorella vulgaris* under IPU influence beginning at 50 µg/L a.i.. Changes in these parameters were not investigated for the algae in the presented study, but the outcome of the cited study hints at effects that influence the quality of the algae as nutrition for grazers. Even under uninfluenced conditions, bigger algae hinder grazing (SOMMER 1994). The most abundant algae, *Cryptomonas ssp.*, has lower ingestion rates than other algae even under normal conditions (INFANTE 1973), mainly due to its big size. When it is getting even larger with IPU treatment (as seems possible due to the results of RIOBOO *et al.* 2002), its nutrition quality for the zooplankton is poorer. As a consequence, there is an influence not only on

mortality (as in top-down control by predation), but also on reproduction which may well be reduced for energy reasons (HEBERT 1982).

In short, abundance of the grazers may be affected by less reproduction in the lowly treated ponds (yet no effect on the algae's abundance but on food quality) and additionally by an increased mortality in the higher treated enclosures (food limitation). In any case, the zooplankton was found to be more sensitive to the IPU treatment than the phytoplankton. –

Species richness in the zooplankton was reduced in relation to the IPU treatment until day 111 a.t.. No effects in this parameter were seen up to 16 µg/L IPU. -

A model graphical interpretation of the interactions in the ecosystem under IPU influence is presented in Figure 74.

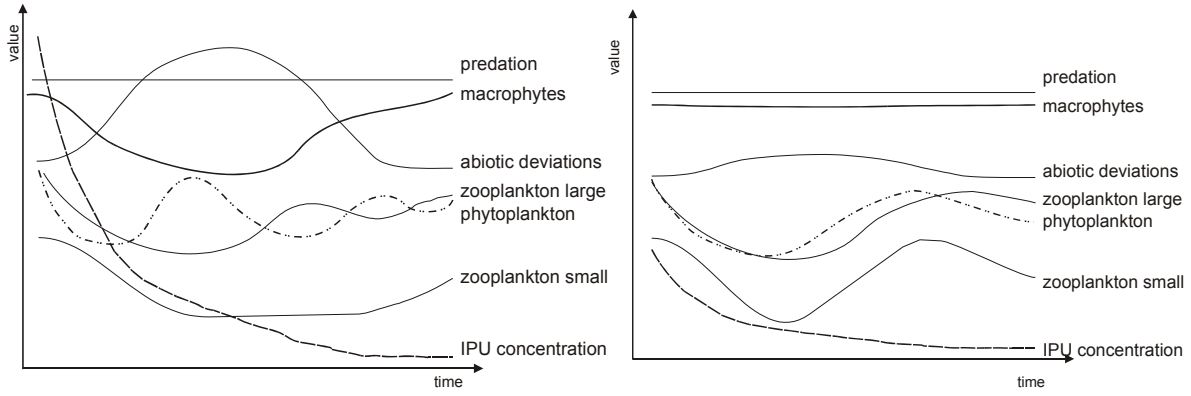


Figure 74: Ecosystem reaction on IPU treatment; left: high IPU, right: low IPU application

The diagram on the left side illustrates the case if macrophytes were reduced by the treatment (IPU4 and 5), the one on the right side if they were unaffected (IPU1-3). The curves are presented in order to clearly see their progression; their actual "value" is not related to any measured parameter (e.g. biomass) and may not be compared directly. Time-dependent reactions and differences/relations in the curves' progression are depicted. Note the higher peaks and oscillations when macrophytes are affected. This pattern can also be seen in the other case, but it is merely a single wave. Deviations in the small zooplankton may be exaggerated a bit if the treatment level is lower than 64 µg/L.

The over-all NOEC for the IPU study is 4 µg/L IPU. Treatment caused intensive food web effects that were affecting the whole pond ecosystem for over one year.

5 Results and discussion of the combined treatment study

5.1 Pesticide residues

5.1.1 Insecticide (CYP)

As mentioned in 2.4.1c the analysis of CYP residues was restricted to the 6 h a.t. and the 3 days a.t. samples because of the problems with the storage of the samples. Analytical data is given in Table 37. Values are not well met and huge losses must be noted. This may be due to the characteristics of the substance (cf. AGNIHOTRI 1989). By defrosting and filling the sample to new bottles the adsorbed part of CYP is lost. However, the mean value of CYP that was found after 6 h a.t. (approx. 21%) is in the lower range that SANDMANN 2000 was able to find after the same period of time without methodical problems. Detecting the correct amount of CYP seems to be rather complicated.

Table 37: Data of the CYP analysis [µg/L] in the combined study

days	level 1	level 2	level 3	level 4	level 5	CYP in [µg/L]
planned	0.015	0.075	0.375	0.750	1.875	
0.4	0.007	0.018	0.118	0.02	0.157	
0.4	0.005	0.016	0.02	0.025	0.1	
3	n.n.	0.006	0.009	0.011	0.026	
3	n.n.	n.n.	0.004	0.016	0.035	
% loss of theoretical concentration						**mean**
0.4	60.0	77.3	81.6	97.0	93.1	79.0
3	100.0	96.0	98.3	98.2	98.4	98.1

In 2000 (CYP study) approx. 38% of the initially planned values could not be retrieved after 6 hours. Here the loss is roughly twice as high. This corroborates the interpretation that a noteworthy amount of the insecticide has been adsorbed into the glass of the bottles (samples filled up twice resulting in a doubled loss). Information about the actual amount of the insecticide was tried to be gained from the biomonitoring experiments (5.2) by looking at the survival of *Chaoborus crystallinus.*

Since application took place in exactly the same way as in the single substance study, a correct treatment can be assumed. Values measured chemically there suggested that the planned treatment levels were reached. All deviations in the biological data in the combined study (see below) that can be addressed to a direct CYP impact were in the same range as in the single application study. GIDDINGS *et al.* 2001 found the results of seven different mesocosm studies with cypermethrin on two continents over a decade remarkably consistent. Therefore, together with the findings presented in 5.2.3, correct dosing can be assumed even without an exact chemical verification.

5.1.2 Herbicide (IPU)

About 98% of the herbicide could be recovered using 100 mL of the sampled water and 96% using 200 mL. The limit of quantification (LOQ) was about 0.1 µg/L, the limit of detection (LOD) 0.05 µg/L. Data is presented graphically in Figure 75.

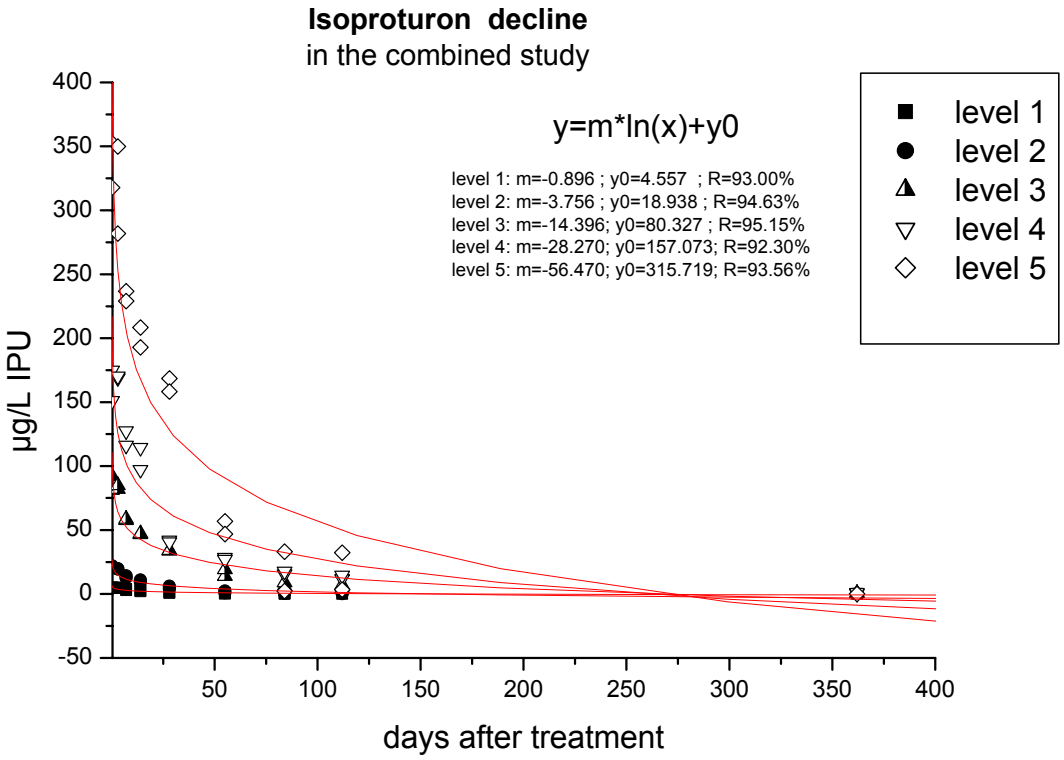


Figure 75: IPU amounts and regression data in the combined study

Table 38 summarizes the results of the regression analysis on the IPU data. Theoretical values were met very well; there was slightly too much of the a.i. in the enclosures, but the deviation is still viable with about 20%. Regression analysis worked very fine, the mean regression coefficient was about 94% and all analysis were highly significant ($p<0.0001$).

Table 38: DT_{50} values and concentrations regression data of the herbicide (combined study)

	level 1	level 2	level 3	level 4	level 5	mean±standard dev.
y0 [µg/L]	4.56	18.94	80.33	157.07	315.72	
y0 planned [µg/L]	4	16	64	128	256	
% of planned	114.0	118.4	125.5	122.7	123.3	120.8±4.6
m	-0.90	-3.76	-14.40	-28.27	-56.47	
R [%]	93.00	94.63	95.15	92.30	93.56	93.7±1.2
SD	0.75	2.73	10.22	25.89	46.80	
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
DT_{50}	12.7	12.4	16.3	16.1	16.4	14.8±0.9
time for detoxification [days]	159	154	265	259	268	

The DT_{50} values were not significantly different from the single application study (p=0.6 in a double side t-test using MS Excel). They are 15 days and 16 days, respectively. The herbicide's total disappearance from the water column was a little big quicker, though. In level 1 and 2 it took a little bit more than five months and in the others about 9 months (about one year in IPU alone). This development may be due to a more intense growth in macrophytes (5.4). MERLIN *et al.* 2002 found bioconcentration in plants to be one of the most important factors involved in the IPU decay. Additionally, higher water temperatures may have led to a faster biodegradation (5.3.6).

5.2 Biomonitoring

Effects of IPU in the combination experiment are not expected, because there were none to be seen in the single substance study (IPU alone). The herbicide did not negatively affect the tested invertebrates directly. Data generated here can be compared directly to the single CYP application. Note that all concentrations or pesticide data referred to in the following are CYP values.

5.2.1 Simocephalus vetulus

Data of the monitoring with *Simocephalus vetulus* is presented in Table 39. Chi-squares indicate that all the data in the analyses were not significantly different from the regression model used.

Table 39: Regression analysis of biomonitoring on *Simocephalus vetulus*

S. vetulus	6 h a.t., 24 h		6 h a.t.,72 h		7 days a.t., 24 h	
parameter	value	error	value	error	value	error
Chi^2	1.822		1.730		0.553	
start (A1)	10	0	10	0	9.13	0.27
end (A2)	0	0	0	0	0	0
LC50 [µg/L] (x0)	1.032	0.181	0.041	0.010	0.929	0.100
order (p)	1.658	0.495	1.390	0.404	1.799	0.335

LC_{50} values are not too consistent. The value for 6 hours a.t., 24 h, is about 1000 ng/L CYP and thus much higher than in the laboratory (500 ng/L) or in the biomonitoring of the single substance study (140 ng/L). At about 72 h time data is matching a little bit better: 41 ng/L here and 17 ng/L a.i. in the CYP study, respectively.

The ratio is reversed for the 7 days, 24 h data. There was no effect in the CYP study, but here a LC_{50} of 930 ng/L a.i. was calculated.

All the biomonitoring tests were performed at Grünschwaige research station. Conditions were not standardized for temperature, light, or cultivation/life history of the animals. These parameters, among others, may lead to the noted deviations.

I therefore strongly suggest using cultivated animals for biomonitoring tests like the ones performed here, especially when the animals are not too sensitive towards the pesticide.

5.2.2 Eudiaptomus gracilis

Table 40 lists the results of the experiments with the calanoid Copepod. All water used here was taken 6 h a.t.. LC_{50} for 24 h is higher than the highest concentration in the test and cannot be interpreted any further. The animals were not very sensitive in this exposure time. After 72 h there is a value of 490 ng/L. Single species test in the laboratory (see CYP part) provided LC_{50} values of about 700-800 ng/L for up to 48 h exposure times. A lower value after a longer period of time is sensible and even the order of magnitude matches well.

Table 40: Regression analysis of biomonitoring on *Eudiaptomus gracilis*

Eu. gracilis	24 h		72 h	
parameter	value	error	value	error
Chi^2	0.20		0.50	
start (A1)	10	0	10	0
end (A2)	0	0	0	0
LC50 [µg/L] (x0)	5.415	2.34	0.487	0.029
order (p)	1.279	0.42	3.250	0.561

The Copepod is thus comparably sensitive in the biomonitoring as in the laboratory test. In the CYP study, *S. vetulus* was more susceptible than *Eu. gracilis*. This result was confirmed here, even though the Cladoceran was less sensitive in the combined biomonitoring than in the other analyses. A comparison of the LC_{50} data is given in Table 41.

Table 41: Comparison of the sensitivity of S. vetulus and Eu. gracilis towards CYP

test	S. vetulus LC50 [ng/L]	Eu. gracilis LC50 [ng/L]
6 h a.t., 24 h	1032	higher than the test range
6 h a.t., 72 h	41	490
single species lab test	approx. 500	700-800

5.2.3 Chaoborus crystallinus

The insect larvae were the most sensitive endpoint in the insecticide study. LC_{50} was about 13 ng/L and thus lower than the test range. An LC_{50} of 15 ng/L was found in the lab (FUNK and HUBER, personal communication). In Table 42, LC_{50} values for several dates are calculated using the initially planned amount of CYP. Please note that the DT_{50} of the insecticide is about 2-3 days. Consequently, the values of the a.i. that can be expected in the water column after 7 to 35 days are lower than the initial amounts. The LC_{50} data calculated with the initial concentrations are therefore higher than expected.

Table 42: Regression analysis of biomonitoring on *Chaoborus crystallinus*

Ch. crystallinus	7 days		20 days		28 days		35 days	
parameter	value	error	value	error	value	error	value	error
Chi^2	0.042		1.069		1.456		0.756	
start (A1)	10	0	10	0	10	0	10	0
end (A2)	0	0	0	0	0	0	0	0
LC50 [µg/L] (x0)	0.090	0.002	0.633	0.372	0.998	3.523	24.343	41.488
order (p)	16.45	$1.62*10^{-6}$	1.282	0.579	0.681	0.535	0.408	0.191

On day 41 a.t. the insect larvae were not affected any more and regression analysis was not possible. This is corroborated by the findings in the CYP study. The highly treated ponds had lowered abundances in *Ch. crystallinus* up tp day 41 a.t..

The LC_{50} data was used to calculate estimations of the actual concentrations of CYP in the water on the sampling dates. Therefore, the LC_{50} value from Table 42 were divided by the laboratory LC_{50}. Thereby a factor for each day is created. By dividing the initially planned concentration with this factor, an estimation for the concentration in the water is computed. The results of this computation are listed in Table 43.

This procedure implies that the "real" LC_{50} for the taxon is constant and has the same value in the biomonitoring and in the lab. The very high sensitivity of *Chaoborus* towards the agent supports at least the latter premise by leveling out differences in the testing conditions. Moreover, the conditions in this biomonitoring experiment were comparable to the single species test to some extend. Pond water and animals from unaffected ponds of the facility were used in both types of studies. The main difference is the use of a climate chamber in the lab, but the impact of CYP on *Chaoborus* is very fast. Animals normally die within the first few hours a.t.. The biomonitoring experiments were evaluated 24 h after their start. During this rather short period of time, differences in living conditions may not have a major influence.

Table 43: CYP concentration estimations by the results by *Chaoborus* monitoring

LC_{50} ng/L (laboratory)		15			
days a.t.	**0**	**7**	**20**	**28**	**35**
LC_{50} (time,real) [ng/L]		89.7	633.3	997.8	24342.5
factor (by the day)		6.0	42.2	66.5	1622.8
c (CYP) [ng/L]					
level 1	15	2.5	0.4	0.2	0.0
level 2	75	12.5	1.8	1.1	0.0
level 3	375	62.7	8.9	5.6	0.2
level 4	750	125.4	17.8	11.3	0.5
level 5	1875	313.5	44.4	28.2	1.2

Regression analysis on the data in Table 43 was done using the model $y=y0*e^{(-a*x)}$. The regression coefficient and the value of "a" is equal for all treatment levels, of course: 93.49% and 1.855, respectively. Further data is presented in Table 44.

Table 44: Initial amount of CYP by *Chaoborus* monitoring

CYP amounts	level 1	level 2	level 3	level 4	level 5
y0 [ng/L]	13.78	68.88	344.39	688.77	1721.90
planned [ng/L]	15	75	375	750	1875

The planned values are met quite well. 91.8% of the planned values were reached. This is the same for all cases due to the computation. The findings are supporting this kind of "biological concentration measurement". The quality is very much depending on the laboratory data. Using values other than 15 ng/L as the LC_{50} of the lab, the concentrations are not met that

well. DT_{50} for the computed concentration data is 3.28 days. This matches the 2.36 days of the single substance study.

In short, is was possible to deduced convincing data on CYP concentrations and decay with the biomonitoring data. Since application took place in exactly the same way as in the single substance study, a correct treatment can be assumed.

5.3 Water quality parameters

For all the following parameters, NOECs, NECs and other treatment related parameters are given in such a way that only the pesticide that exerted the bigger influence in the single application studies is given as a reference. The other one is omitted to make it easier to read. Please note the way the NECs are determined. They must be related to a metric axis (proportionally scaled). The "levels" cannot be used for this, because the relation between the two pesticides is not constant. Consequently, the toxin with the bigger influence in the single application approach was used. Whenever there are hints that a combined action may lead to the influence, the "level" that is related to this amount of a.i. is noted. Applied pesticide amounts are listed in Table 45. They are identical to the amounts used in the single pesticide approaches.

Table 45: Treatment levels in the combined study

Level	amount IPU [µg/L]	amount CYP [µg/L]
level 1	4	0.015
level 2	16	0.075
level 3	64	0.375
level 4	128	0.750
level 5	256	1.875

The water quality parameters measured will now be discussed in greater detail.

5.3.1 Alkalinity

Alkalinity was influenced by the combined treatment. The progression is given in Figure 76. Values in level 3-5 start rising shortly after the application; the earlier and more pronounced the more of the pesticides were used. Level 2 shows no effect at all and level 1 only minor deflections in autumn 2001.

Effects are compensated by June 2002 at the latest, about one year a.t.. NOEC for the parameter is level 2 (n=11 starting on day 14 a.t.). A comparable impact was seen in the IPU study. In the combination, there is a conjunction of the recovery in alkalinity with the loss of the herbicide from the water column. So this effect is predominantly triggered by IPU. A NOEC of 16 µg/L IPU in the combination can be noted. In the IPU study it was 4 µg/L.

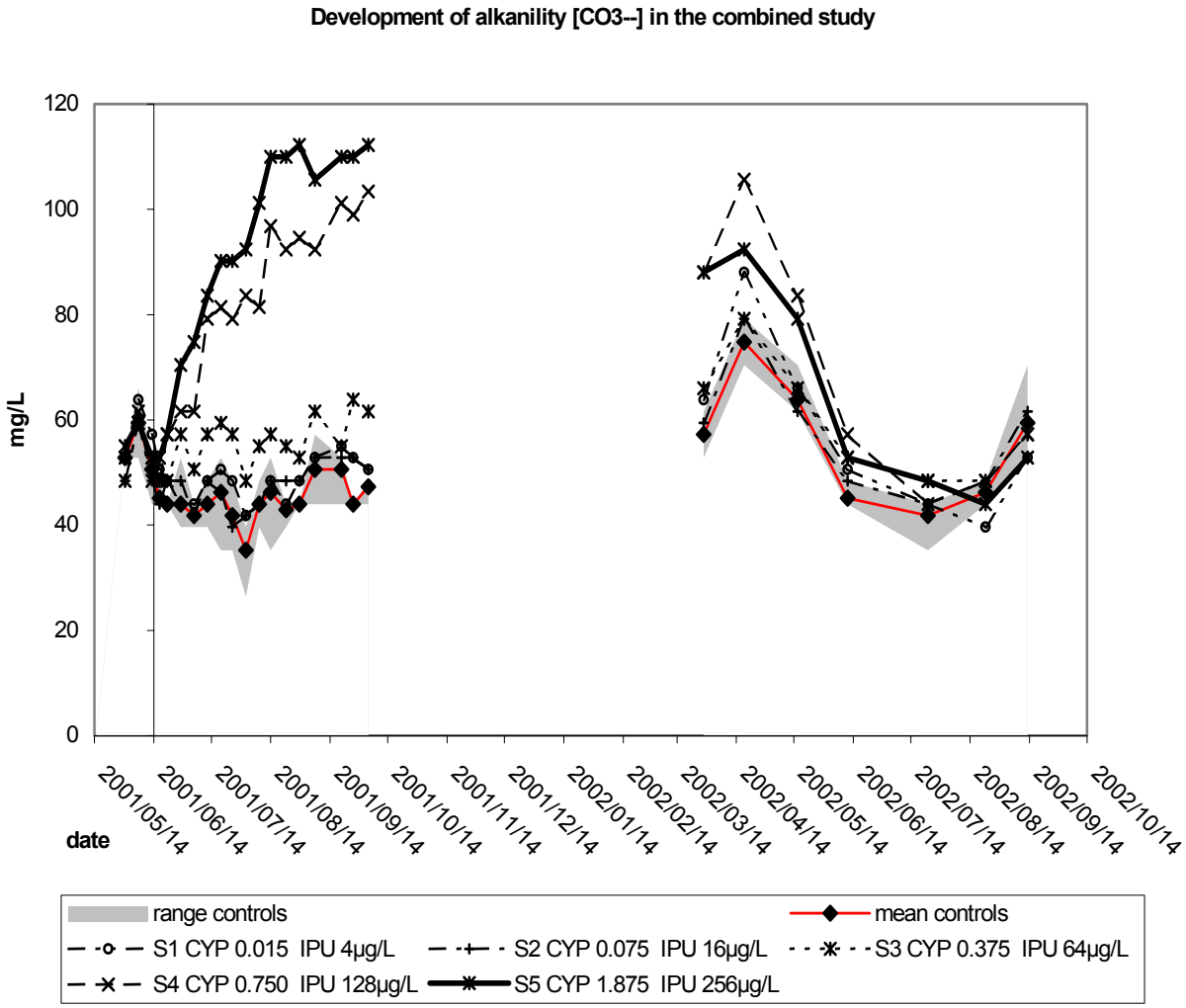


Figure 76: Alkalinity in the combined study

NECs for IPU in the combination are 13.2-19.6-29.2 µg/L (n=2) and thus lower than in the IPU study (about 40 µg/L IPU as NEC). Hence, the ecotoxicological endpoints NEC and NOEC are closer to each other in the combined application study. Additionally, they are both higher than the NOEC of IPU alone (CYP showed no effect).

This finding may be due to an interaction of the macrophytes and the algae on this parameter. Macrophytes set the basis of the parameter, proven by the recovery time of the alkalinity that is related to the re-growth of the macrophytes. Major deviations from the controls starting in level 3 follow alterations of their cover. Level 3 is the first treatment level to show impact in the submersed plants (cf. 5.4). The algae add the finer fluctuation on the general trend that is determined by the macrophytes. The crucial question then is why level 2 does have statistically significant treatment effects (on the alkalinity) in the IPU study but not in the combination[20].

[20] Please note: Macrophytes were affected at IPU4 and higher with IPU treatment alone and from level 3 on in the combination. Alkalinity should be affected at corresponding levels, too (i.e. influenced at a lower level in the combination). Interestingly, the relation is reversed. As noted above, changes in the algae may be a reason for this.

First of all, NOEC and NEC of the IPU study bracket these parameters together in the combination[21]. Since the comparison of NEC and NOEC (IPU to combined approach at each case) contradict each other, it is not too sure whether there is a difference in the impact at all. IPU2 is well within the control range all the time, so the NOEC derived by Williams' test may be too high (although there is an increasing trend in autumn 2000, cf. the IPU part of this thesis, 4.4.1). Assuming that IPU2 level has no effect either, the NOECs are identical and all impact on alkalinity is solely due to the IPU amounts in the combination.

Nevertheless, a biological interpretation assuming that the combination is indeed less sensitive (based on the NOECs and no obvious trend in level 2) can be given:

With IPU alone, the algae's physiological performance (for example photosynthesis) is reduced at IPU levels higher than 4 µg/L. Evidence is given by the oxygen content (4.4.3). It can be assumed that this loss in performance also occurs in the combined approach (cf. 5.3.3). Consequently, effects of an altered physiological performance can be assumed starting in level 2. This may eventually lead to an impact on alkalinity, because it is linked to the metabolism of photo-autotrophic organisms (SCHWOERBEL 1999). Please remember that level 1 shows no effect on alkalinity in both types of study[22], but level 2 does only with IPU alone (it is not influenced in the combination). A rationale may be given by the interaction with the zooplankton. Reduced grazing (DAY and KAUSHIK 1987, FERNANDEZ-CASALDERREY, FERRANDO *et al.* 1994) is a possible sublethal effect on zooplankton in combination treatment level 2. The treatment with low amounts of CYP hinted at such effects in the CYP study. Phytoplankton abundance is not altered by the combined treatment in level 2 (5.5) or by the treatment with 16 µg/L IPU (which is also the $NOEC_{community,\ Phytoplankton}$ in the IPU study). Consequently, the reduced grazing does not trigger a numerical reaction in the combination study (for the total abundance). However, a qualitative one can indeed be found (Figure 88 and Figure 87): More Chrysophyceae are present in level 2 due to the combined treatment than in the controls in the first year of the combination study[23]. A different class distribution and simultaneously the same total abundance of algae may lead to differences in the physiological reaction that influences the alkalinity, i.e. a community with more Chrysophyceae is performing the same impact on alkalinity as the controls do with less of these algae. Treatment with IPU alone did not shift the community (grazing can be assumed as constant) and effects on alkalinity become visible.

In other words: The herbicide treatment did not change the algal community at 16 µg/L IPU (single a.i.). The physiological reaction is reduced and thus alkalinity is altered. Combining

[21] For convenience of comparison:

in amount of IPU	IPU study		Combination
NOEC	4 µg/L	<	16 µg/L
NEC	40 µg/L	>	20 µg/L

[22] Minor deflections from the control range are most probably due to differences in the test systems. Findings in the single pesticide studies do not hint at toxic effects of any kind in this level. Additionally, level 2 (combined treatment) is even better inside the control range. There is no indication why the combination of the lower concentrations should be influenced more than the higher ones.

[23] The "trouble" with the higher NOEC in the combinations is derived from data of this particular year.

16 µg/L IPU with 0.075 µg/L CYP (i.e. level 2 in the combination) does alter the algal community structure (secondary reaction to reduced zooplankton grazing). This "new" phytoplankton community is able to keep up with the physiological performance of the controls and thus no effect on the alkalinity is visible in the combination.

Additionally, the development described above may also be promoted by some differences in the macrophytes between level 1 and level 2. Please refer to chapter 5.4 on page 146 for details.

In this way, alkalinity could be considered less sensitive to the combined treatment than to the herbicide alone. If such an effect also holds true for the other water quality parameters, a combination effect might be noted.

5.3.2 Conductivity

This parameter is developing in the same way as the alkalinity. Level 3-5 has increased amounts of ions in the water (Figure 77). Maximum values are about 250 µS/cm, thus somewhat lower than in the IPU study (around 290 µS/cm). Recovery in level 4 and 5 takes about one year; in the other enclosures the parameter is back to "normal" in spring 2002 (second year). All these times relate to the time for the IPU decay (5.1.2).

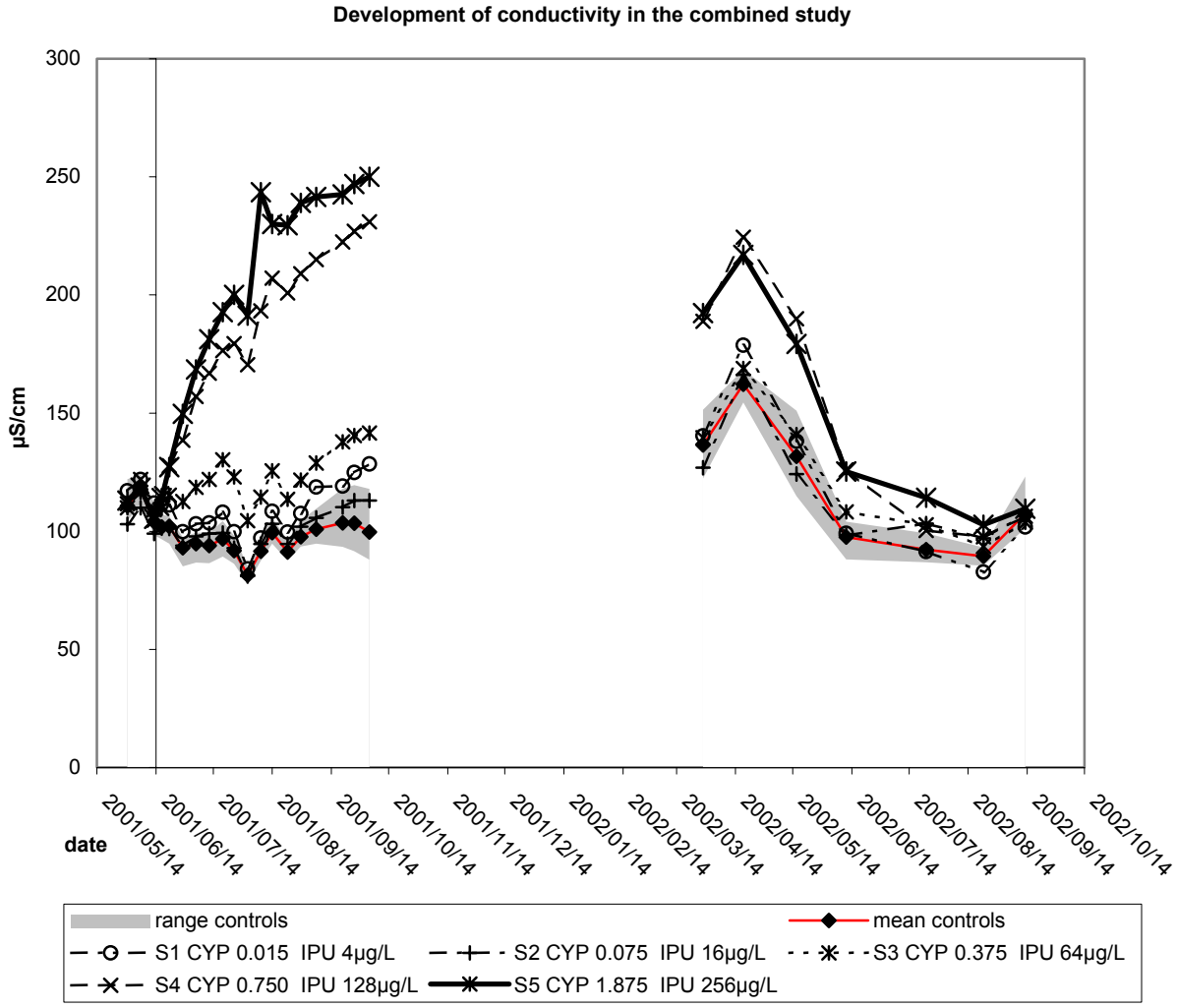


Figure 77: Development of the conductivity in the combined study

NOEC is level 2 in the combined study (n=13) beginning on day 14 a.t.. The NEC values calculated for IPU in the combination are 14.8-17.2-20.0 µg/L (n=3) and are strongly backing the NOEC. NOEC in the IPU study is lower (4 µg/L), whereas NEC is higher (about 40 µg/L). In the combined approach, level 1 has a higher conductivity than level 2 most of the time but does not leave the control range too far. The reason for this could be the slightly different macrophyte stock in level 2 (see 5.4). By a higher uptake of ions conductivity can be lowered. Another (or additional) reason might be that the two enclosures treated with the level 1 dose are simply somewhat different from the level 2 enclosures by chance events (for example the sediment composition).

However, a linear trend starts in level 2[24], which is consequently the NOEC. Interestingly, the NEC in the combined study is lower than the NEC in the IPU approach. In the latter case, NECs were quite high, mainly due to bigger variations in the controls. Even so, the lower intersection with the 95% confidence interval was higher than the highest value in the combined treatment, 42 µg/L and 20g/L IPU, respectively.

Effects of the combined treatment can thus be regarded similar to the single IPU application. Alternatively, an interpretation following the one given with alkalinity could be taken into account, so that the conductivity may even be less sensitive in the combination treatment.

5.3.3 Oxygen content

Oxygen contents are depicted in Figure 78. All other measured oxygen parameters did not show a different development (data not shown). Distinct decreases were found for Level 3 to 5 in the first year. NOEC is level 1 (n=2, days 3 and 7 a.t.). In March 2002 all values are in the same range. The oxygen content may be influenced by both pesticides. The impact of IPU alone is well defined (cf. 4.4.3). In the CYP study, the oxygen content was not varied by the treatment. A combined action could be that less zooplankton (due to CYP) allows more algae to survive the IPU treatment. Consequently, oxygen could react less sensitive to the combined treatment than in the IPU study. Actually, this is not the case[25]. NOECs are identical. NEC is 11.2-13.5-16.3 µg/L (n=11) for IPU in the combination; again in line with all the NOECs and the NEC of the single application study (IPU) for this parameter.

However, the inversion between level 1 and 2, like the one in the conductivity, can also be seen here but to a lesser extend. The mean oxygen contents in level 1 and 2 are almost identical on day 3 and 7 a.t.: 9.8 mg/L in both levels on day 3 a.t. and 9.6 and 9.5 mg/L, respectively, on day 7 a.t.. These values lead to the low NOEC of level 1. Since the values in oxygen on these sampling dates are so close to each other, the NOEC could well be level 2 and the lower one is thus only a statistical artifact. NOEC for day 14 to 41 a.t. is level 2.

The biological background for a NOEC of level 2 is given in 5.3.1 (alternative hypothesis).

[24] without that this level is significantly different from the controls in the Williams' test ($p>0.05$).

[25] Combined treatment alters the class distribution in level 2 but not the abundance of the phytoplankton; see 5.3.1 and 5.5.2. At higher treatment levels the effects of the pesticides on the susceptible taxa outbalance any combined effects.

Another influence CYP has in the combination is, most probably, the elimination of the oscillating pattern of IPU alone. This will be discussed in detail with the plankton data (see below).

In the second year oxygen contents in level 4 and 5 are increased from April on. Williams' test indicated a treatment relation from June to August 2002 with NOECs of level 3, 2, and 4. Looking at the curves again a no effect level of level 3 (64 µg/L IPU) can be noted.

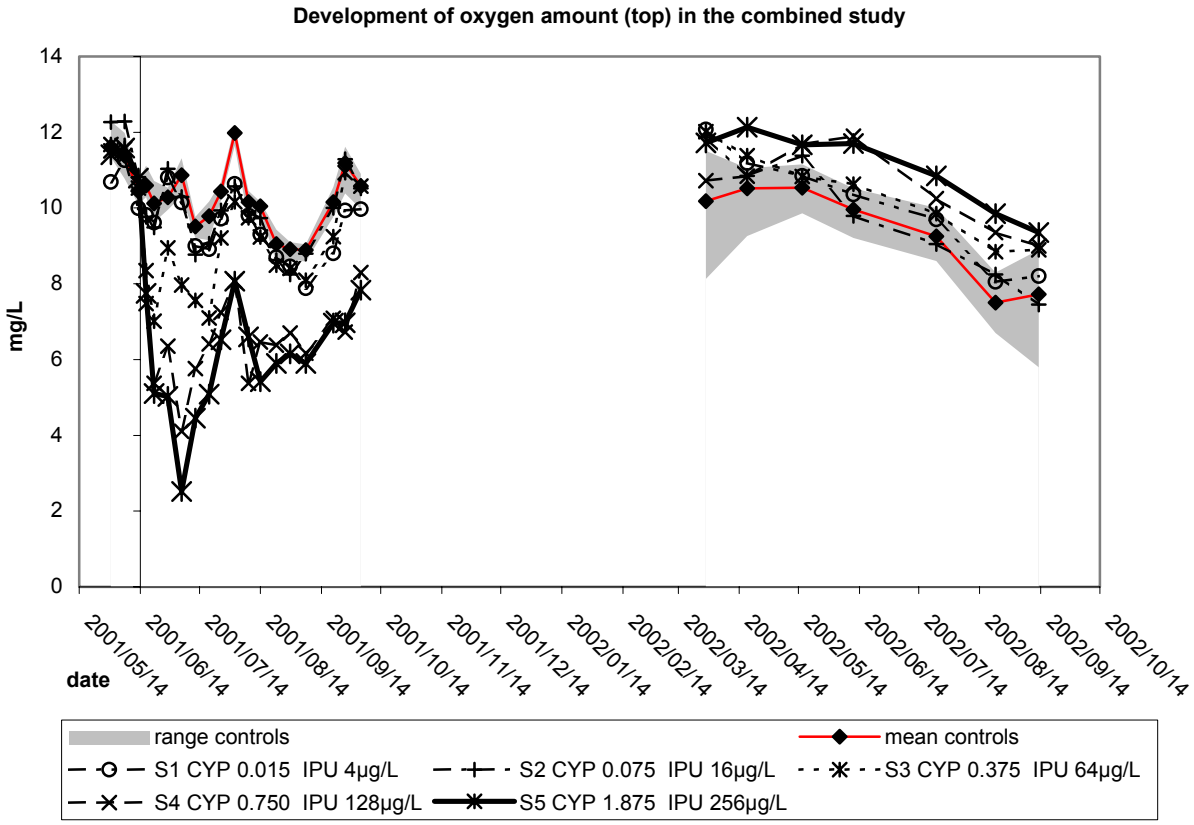


Figure 78: Amounts of oxygen in the combined study

There is an interesting difference between the combined study and IPU alone. In the latter study, oxygen amounts in the enclosures treated higher than 16 µg/L IPU were lower than the control range up to June. In the presented case, treated enclosures have the tendency to be higher right from the beginning of the second year. This could be due to reduced grazing in the first year and therefore better starting conditions for the algae's photosynthesis when the herbicide is degraded.

5.3.4 Chlorophyll a

The pigment content in the water is depicted in Figure 79. Amounts are fairly low, less than 0.2 µg/L most of the time. Increases are seen in level 4 and 5 on days 28 and 35 a.t.. Level 5 is still quite high in pigment on day 41 a.t. but inside the broader control range on that day. A NEC could not be calculated. The increases are well related to higher abundances in the phytoplankton (5.5.2). "Shade adaptation" types of reactions as they were hinted at in the IPU study cannot directly be seen here but can be expected. Increases were also found in the

insecticide study because of reduced grazing pressure in the two highest treatment levels. The increase here is more distinct than the one in the CYP study. Hence, reduced zooplankton grazing due to the insecticide (see abundance data of the zooplankton, 5.6.2a) plays a more important role for the algae under simultaneous herbicide action than without it. This is a clear combination effect.

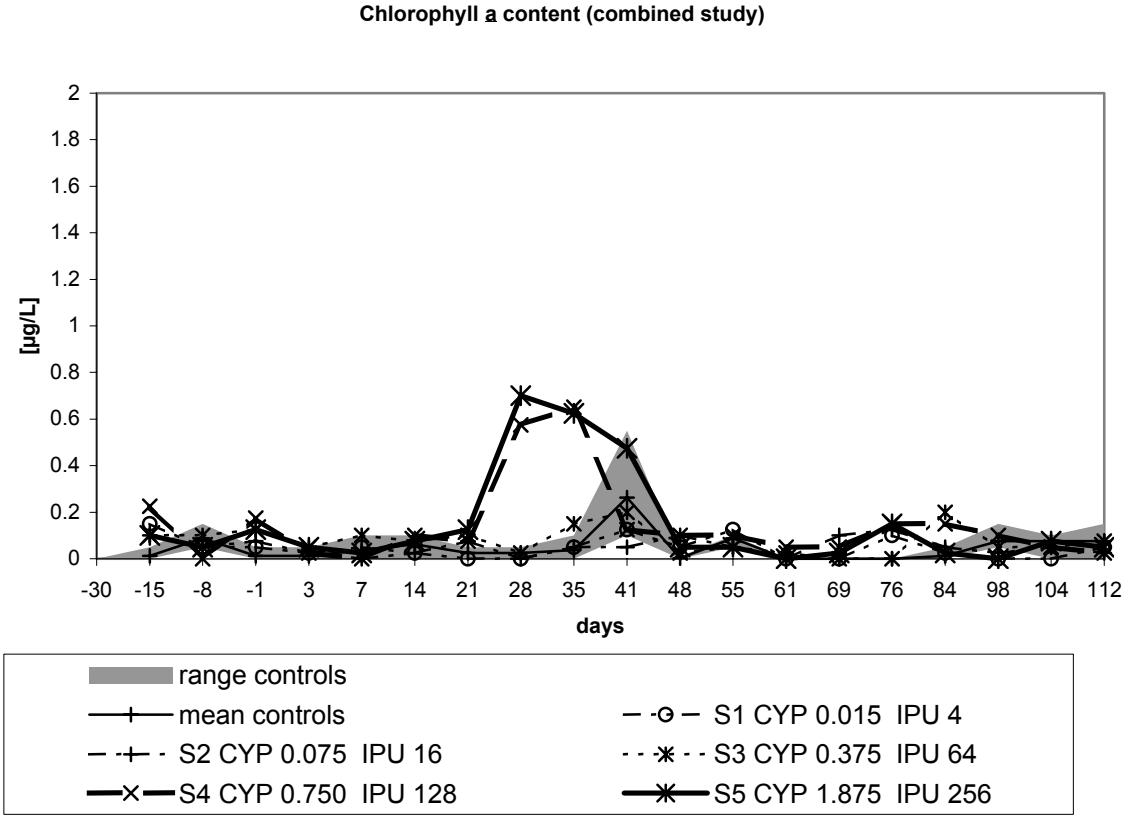


Figure 79: Photosynthetic active chlorophyll a in the combined study

5.3.5 pH value

The pH in the water is clearly treatment related (Figure 80). In the second year, only minor deviations were visible that could not be related to the treatment. Interestingly, level 1 is lower than level 2 and even the controls all of the time. This difference existed already before the application and the curves are almost parallel. A treatment effect can be excluded. Differences in the test systems, as discussed above, most probably trigger this development.

The combined treatment affected the two highest levels almost identically. The pH decreases to values of about 7-7.5. In the controls it is at about 9. Level 3 has an intermediate value (7.5-8 up to day 35 a.t. and then 8-8.5). NOEC is thus level 2. NEC is 4.2-16.4-70.7 µg/L IPU in the combination (n=14), indicating the same range. Recovery could be seen after winter.

Alike in the IPU study, this is a class 5 effect (BROCK *et al.* 2000 in EU 2002) in all ponds treated higher than the NOEC.

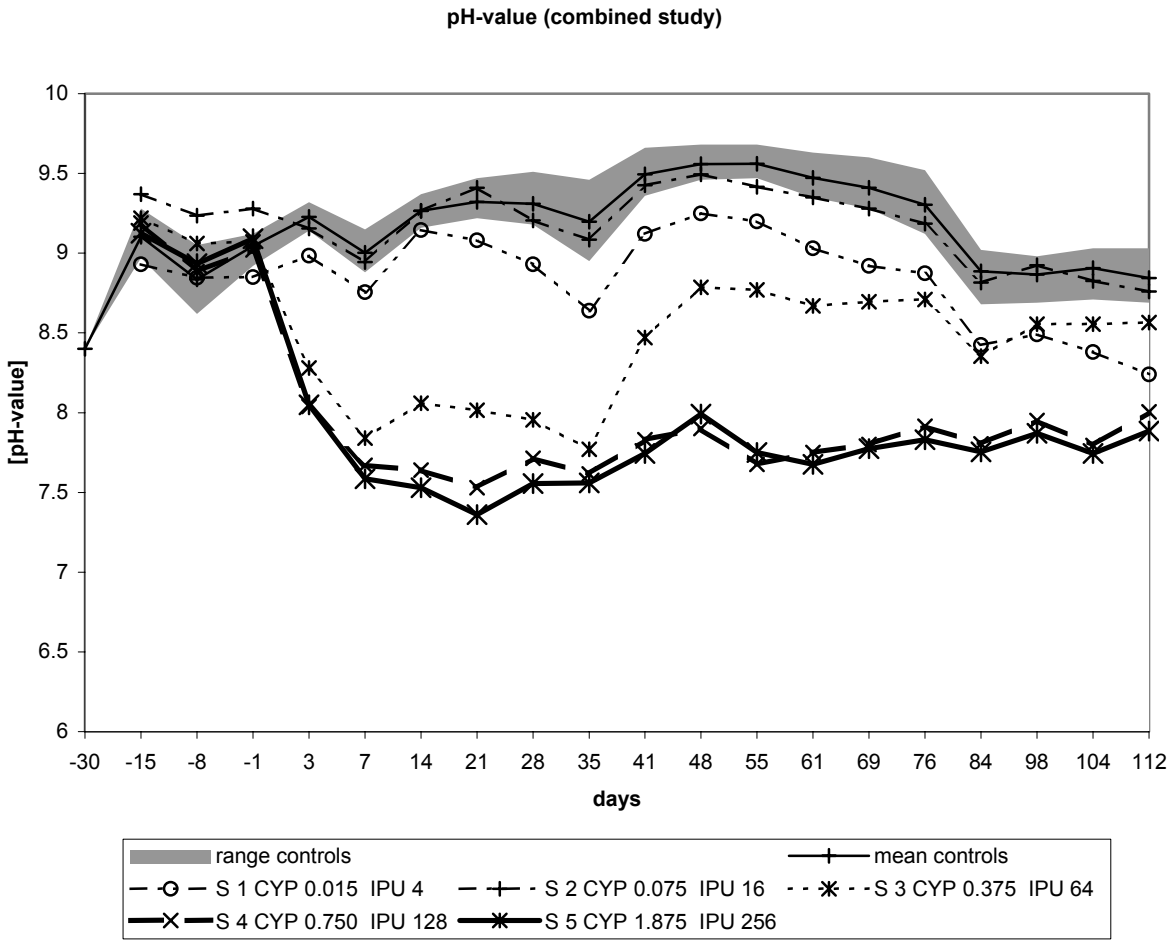


Figure 80: pH value in the combined study

The NOEC here is higher than in IPU alone (4 µg/L). Differences are not due to the inverted pH relation between level 1 and 2 that could render statistics less sensitive. Because the pH of level 2 is in the control range most of the time it is clearly not affected.

In short, pH is less sensitive towards IPU in the combination than in the single application. This may be due to the less pronounced influence of the combined treatment on the algae. The macrophytes seem to set the general level of the systems' pH (and other water quality parameters). Variations in the algae lead to minor deviations, as long as the macrophytes are not severely affected themselves (i.e. they have a stabilizing effect, EU 2002). Since macrophytes have a NOEC of level 2 and IPU3, respectively, alterations in the NOEC of the pH must be due to the algae (which are differently affected in the combination, see 5.3.1 for a rationale).

5.3.6 Temperature

Temperature curves are presented in Figure 81. Please note the warm water in autumn. In the single application studies, temperature constantly fell from July on. Here they remained higher including August and had an increase in October again. This development may have led to a faster decline in the pesticides.

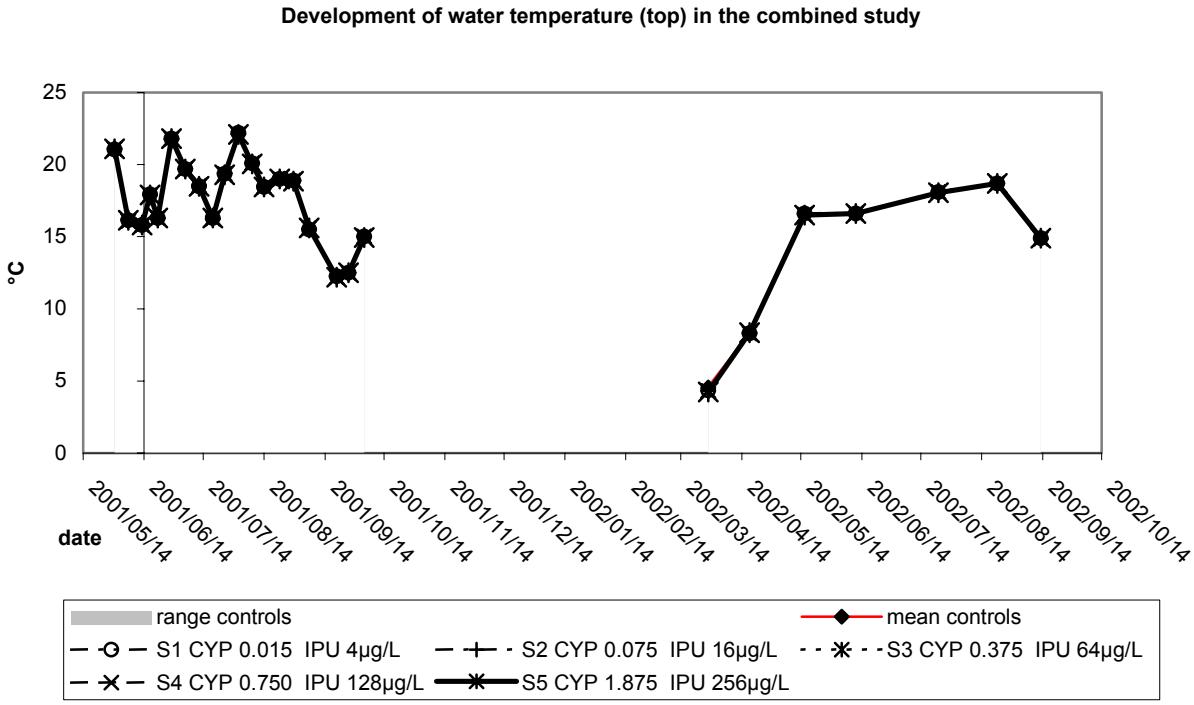


Figure 81: Development of the water temperature in the combined study

5.3.7 PRC analysis

PRC analysis of the water quality parameters showed distinct treatment effects (Figure 82). Recovery can only be seen in level 3 on day 55 a.t.. Level 1 differs more from the controls (x-axis) than level 2. This has already been seen in the conductivity, the dissolved oxygen and the pH and is regarded as an effect of the macrophytes (5.4) and the algae.

This analysis is significant, p=0.005. It explains 55.8% of the variances by the treatment of which 82.9% are displayed. 38.4% of the variations are explained by the sampling day. Table 46 lists the relevant parameters in this analysis.

Table 46: "Species" scores of the water quality parameters in the combined study

parameter	score
alkalinity	0.8414
conductivity	0.8406
O2 saturation top	-0.7389
O2 [mg/L] top	-0.7633
O2 saturation bottom	-0.7635
O2 [mg/L] bottom	-0.7840
pH-value	-0.8766

As in the IPU study, a clear DO-pH-alkalinity-conductivity syndrome (KERSTING and VAN DEN BRINK 1997) can be seen: Alkalinity and conductivity are rising, whereas pH and oxygen are falling with the treatment level.

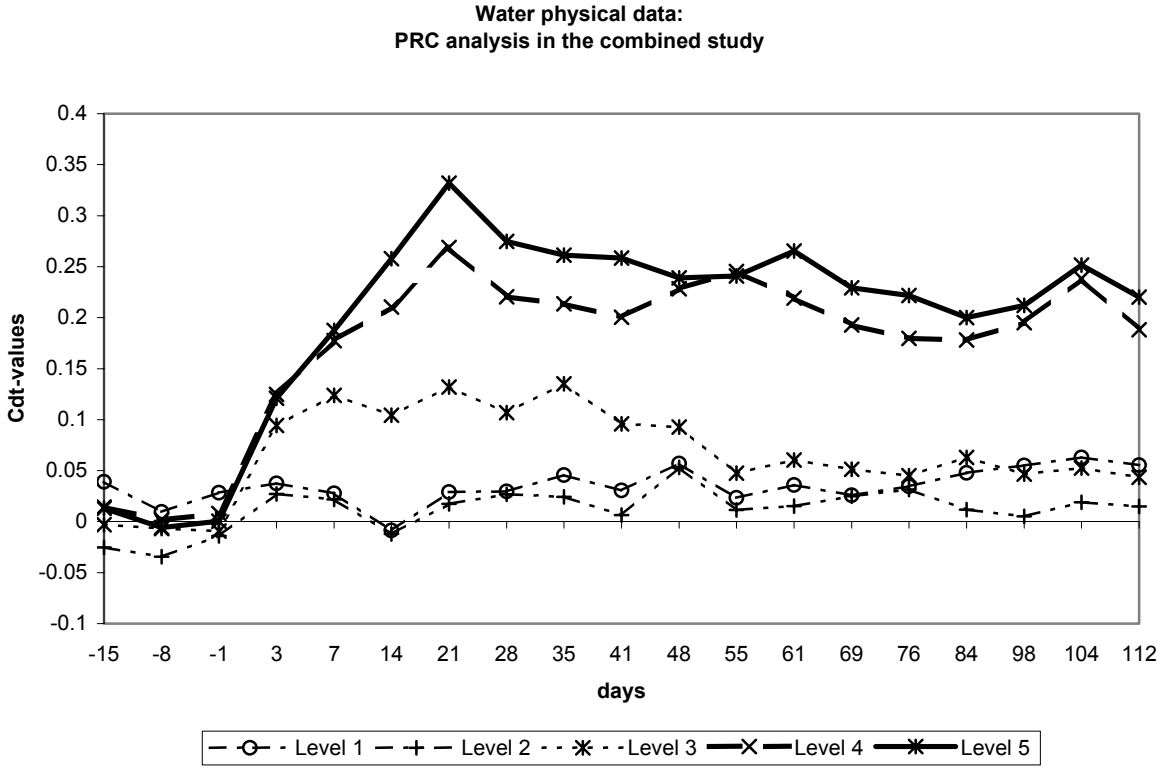


Figure 82: PRC analysis of the water quality parameters in the combined study

The NEC for the PRC is 17.3-19.2-20.9 µg/L IPU in the combination (n=9). In IPU alone the no observed effect level was 4 µg/L; here it is level 2 (16 µg/L IPU). This higher value relates to the different effects on phytoplankton due to the combined treatment. Further discussion on this issue is given in 5.3.1.

5.3.8 Water chemistry

5.3.8a Overview

Water chemistry data is summarized in Table 47. As in the single application studies, this is an oligo-mesotrophic system (SCHWOERBEL 1999). All parameters are in the range of the IPU and the CYP study. No treatment effects on the phosphorous fractions could be observed. The other influenced parameters are presented in greater detail in the following.

Table 47: Summary of water chemistry parameters in the combined study

		mean	std. dev.	min	max
TP [µg/L]	treated	20.45	7.80	8.14	87.28
	control	19.59	8.44	7.95	66.63
SRP [µg/L]	treated	3.41	3.73	0.00	23.58
	control	3.77	3.45	0.00	18.53
NO3-N [mg/L]	treated	0.026	0.015	0.002	0.127
	control	0.015	0.006	0.007	0.028
NH4-N [mg/L]	treated	0.055	0.064	0.000	0.482
	control	0.049	0.058	0.001	0.320
silicate [µg/L]	treated	763.61	628.98	66.17	1980.25
	control	388.59	318.02	27.22	1659.66
Na^+ [mg/L]	treated	2.22	0.39	1.57	3.46
	control	1.83	0.37	0.86	2.75
K^+ [mg/L]	treated	0.11	0.12	0.00	0.76
	control	0.05	0.05	0.00	0.32
Ca^{2+} [mg/L]	treated	12.33	4.45	4.20	32.56
	control	8.78	2.11	5.57	13.07
total hardness [°DH]	treated	3.94	0.82	2.60	7.00
	control	3.02	0.23	2.50	3.50

5.3.8b Silicate

Silicate contents in the water are presented in Figure 83. Variations in the controls are rather high on days 7 and 14 a.t.. A reason for this may be the disturbance of the system by the increased frequency of sampling around the application day. Higher variances were also seen in the single application studies on day 14 a.t..

The treated ponds are above the control range even before the application. Still more increased values (treatment related) are found from day 7 to 55 a.t.. Levels 1 and 2 are not affected clearly and inside the control range starting on day 28 a.t.. Effects are thus restricted to level 3 and higher.

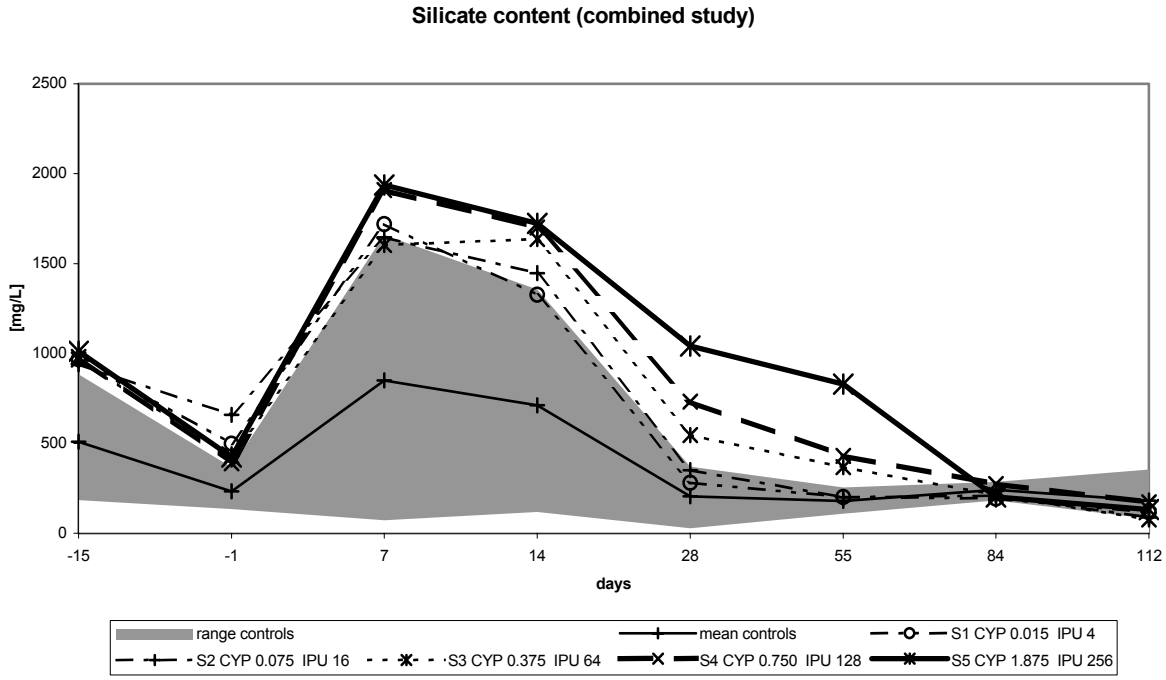


Figure 83: Silicate in the combined study

NEC calculations gave 13.0-17.9-41.7 µg/L IPU in the combination (n=4). This range is in line with ESER 2001 and the results of the IPU study (values between 12 and 100 µg/L).

Differences to the IPU study are the more pronounced reaction in level 3 and the recovery to control values on day 84 a.t.. With IPU alone, IPU treatment levels of level 3 was outside the control range only twice (here three times in a row). Recovery for level 4 was first seen on day 139 a.t. and not at all in level 5. In the combined application, all ponds are close together from day 84 a.t. on.

When interpreting the data, the reaction of the Bacillariophyceae (5.5.2e) must be kept in mind. The planktonic ones show no distinct reaction to the treatment. Variations in the silicate may therefore results from the periphyton as already mentioned in the IPU study. The earlier recovery can be explained in this way: CYP treatment negatively affects macroinvertebrate grazers[26] in the levels in question. IPU concentrations on day 84 a.t. are 7 µg/L to 17 µg/L, that means lower than the NEC for the periphyton (ESER 2001). Being released from grazing to some extend, periphyton can grow and consume the silicate earlier than in the IPU study. Growth may take place on the enclosure walls, because the macrophytes are affected already at these treatment levels and concentrations (5.4).

In short, silicate possibly exhibits special combination treatment effects but the no effect level (regarding IPU) is similar to the herbicide study.

[26]for example EC_{50} (24 h) for *Asellus aquaticus* 0.048 µg/L CYP (ROTH 2001); the $NOEC_{community}$ for the macroinvertebrates was calculated 0.015 µg/L CYP (HUBER *et al.* in an unpublished GLP study)

5.3.8c Nitrogene compounds

NO_3-N content is increased in level 4 and 5 from day 7 to the end of the year. On day 28 a.t. both levels have approximately the same amount, about 0.03 mg/L. Before that day, level 4 is higher than level 5, afterwards the relation is reversed. Level 3 is also increased to some extend (lower than the above mentioned) on days 7 to 55 a.t.. Level 2 is in line with the controls all the time. Level 1 is higher than the controls on day 7 and day 55 to 112 a.t..

Treatment effects may be seen for level 3 and higher. The increases in level 1 are too small to be due to the application; level 2 showing no effects at all corroborates this interpretation. NEC calculations gave no consistent results (higher than the amount of IPU in level 5).

NH_4-N steadily increases in level 4 from day 55 to the end of the year and in level 5 from day 28 a.t. on. All other levels are not affected. NEC is 76.0-174.6 µg/L (n=3).

In the IPU study, distinct effects started in treatment level 4 (no effect level: IPU3). The same is valid for the NH_4-N in the combined study. NO_3-N is a little more sensitive: no effects only up to level 2.

All these effects are related to the macrophytes (5.4). Because they react more sensitive in the combined study, the more pronounced effect in the NO_3-N can be addressed to this development. Combination effects cannot be seen.

5.3.8d Cations: Sodium, calcium, potassium

Sodium concentrations are quite constant, approximately 2 mg/L in all enclosures up to day 14 a.t.. Later on, level 4 and 5 increase (maximum 3.46 mg/L on day) almost identically until day 112 a.t. (end of sampling). Level 3 is rising in sodium from day 55 on, but is always below the higher levels. Level 1 and 2 have only minor alterations from the control range. NEC values are presented in Table 48. LOEL is level 3 but the no effect level is only slightly below it.

The development of calcium ions in presented in Figure 84. A treatment relation is obvious. The increased values of level 1 on the two last sampling dates may be a chance effect, because level 2 does not leave the control range.

Well-defined treatment effects start in level 3 on day 14 a.t. and gain importance to the end of the sampling period. NEC values (Table 48) below level 3 back this interpretation.

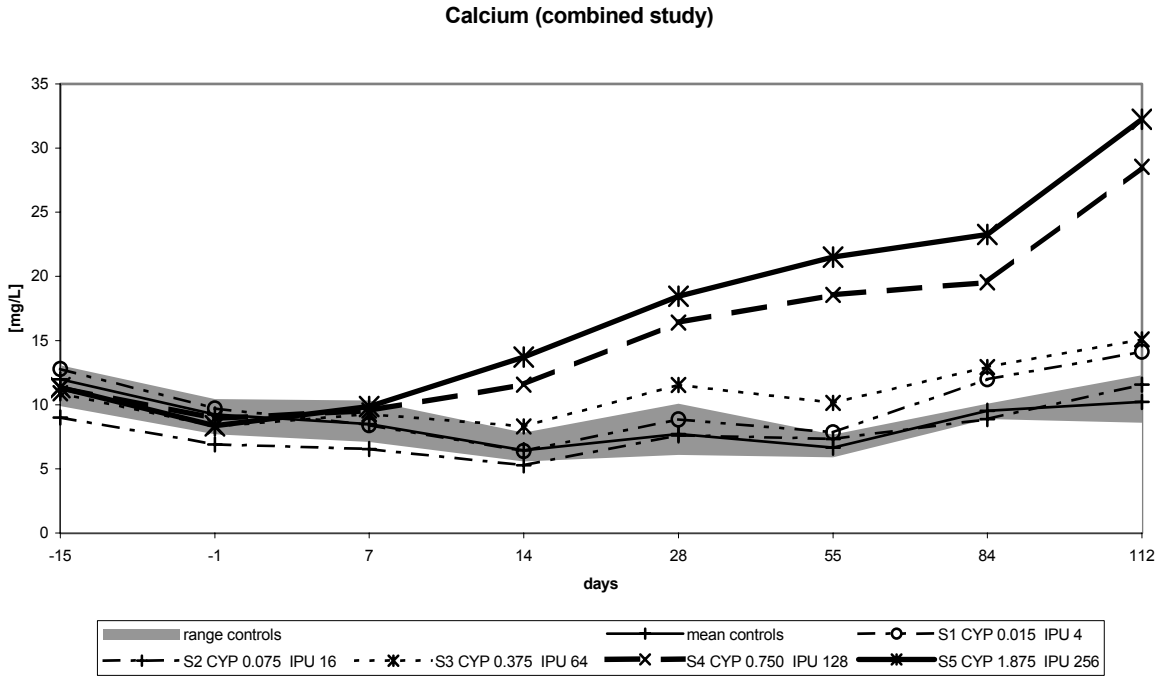


Figure 84: Calcium ions in the combined study

Potassium ions in the water are around or below the LOQ for all treatment levels ≤ level 3 up to day 55. Afterwards, some of the controls reach values of about 0.2-0.3 mg/L.

Treatment effects are seen in level 4 and 5. Starting on day 28 both levels increase parallelly. The maximum is reached on day 112 a.t. (approx. 0.75 mg/L).

NEC calculations (Table 48) may be invalid because of the controls being too near the LOQ. A LOEL of level 4 can be noted, though.

Table 48: NEC values for cations in the combined study

value [µg/L IPU]	NEC Na^+	n	NEC Ca^{2+}	n	NEC K^+	n
upper	115.9	3	51.4	6	91.1	2
middle	55.5	3	26.7	6	65.5	2
lower	26.6	3	16.6	6	47.6	2

Summarizing the cations development in the combined study, effects start in level 3 containing 64 µg/L IPU. The same LOEL was found in the IPU study.

5.3.8e Total hardness

Treatment effects on this parameter are rather pronounced (Figure 85). Increases start on day 7 a.t. in level 3 and get more obvious with time. Recovery cannot be seen at any affected level. Level 1, leaving the control range on day 112, is in line with the development in calcium ions (see above).

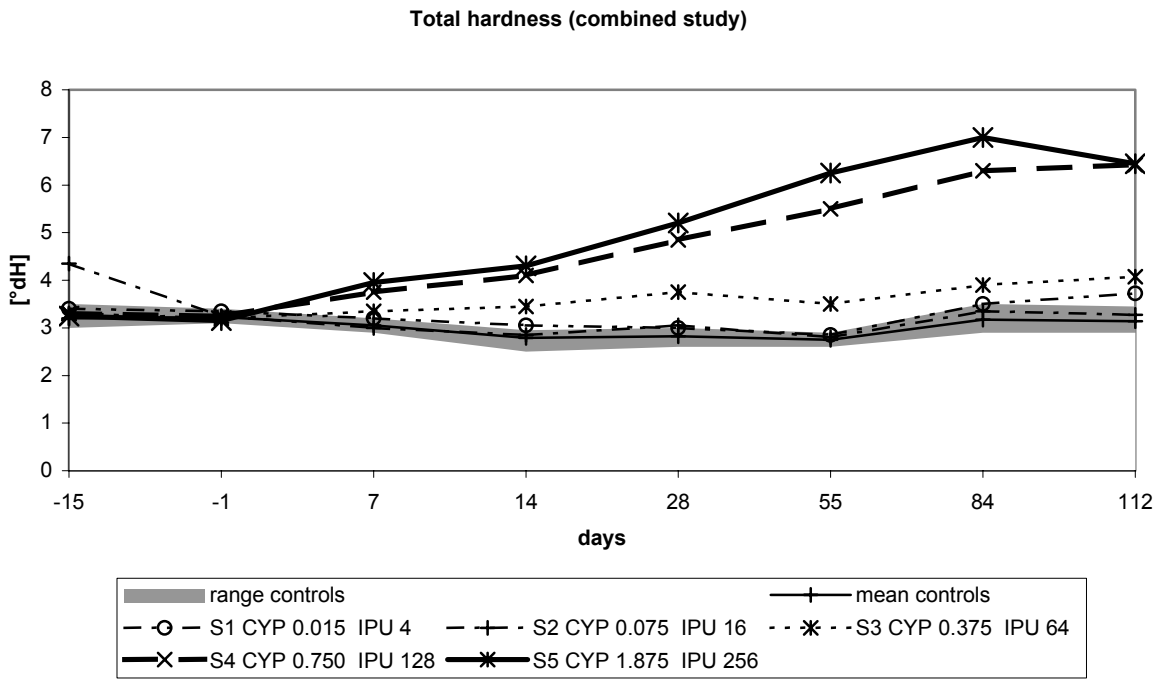


Figure 85: Development of the total hardness in the combined study

NECs are 13.9-17.4-22.1 µg/L (n=6) for IPU in the combination. This is only slightly higher than level 2, but the LOEL is still level 3. In the herbicide study, the LOEL was identical, but the NEC was rather close to IPU3 (about 50 µg/L IPU). The effect in the combination is more pronounced. A reason may be differences between the two pond systems used.

5.3.9 Overview of treatment effects of the combined application on water quality

Effects on water quality parameters were similar to the ones found in the IPU study. A DO-pH-alkalinity-conductivity syndrome (KERSTING and VAN DEN BRINK 1997) was clearly present. NOECs were most of the time higher than in the single application: Level 2 containing 16 µg/L IPU (in the combination) instead of 4 µg/L IPU (alone).

It was possible to explain the higher values by the combined action of the pesticides: By modified grazing pressure on the phytoplankton due to CYP action[27] even in level 1 alterations in the community structure of the phytoplankton at constant abundance took place (higher percentage of Chrysophyceae). Due to this structural difference in the algae impact on the water quality parameters was eventually cut down in comparison to the herbicide study — a distinct combination effect. However, differences in the macrophyte structure between the enlocures that received pesticide dosings of level 1 and 2 may enhance this effect (5.4).

This interpretation only holds true in treatment levels 1 and 2, where the macrophytes were not affected (see 5.4). In the other treatment levels major alteration of the ecosystem took place, like in the IPU study. This was reflected by the time needed for recovery. Control levels

[27] please remember that sublethal effects on grazing were observed in the CYP study; see also DAY and KAUSHIK 1987, FERNANDEZ-CASALDERREY, FERRANDO *et al.* 1994

were reached not before spring of the second year when the submersed plants started their re-growth.

Chlorophyll a related quite nicely to the phytoplankton abundance. Deviations could be seen in an increase in level 4 and 5. This effect was more pronounced than those seen in the single application studies. The distinct increase on two dates may be due to three reasons:

- more pigment in each cell because of "shade adaptation" reaction to IPU;
- less zooplankton on these days as a result of CYP treatment;
- consequently more algae which are not susceptible to IPU.

As seen in the single substance studies, each effect alone is not capable of triggering a very clear impact even at the highest pesticide level. The combination has particularly more distinct consequences.

Water chemical parameters reacted in almost the same way as in the IPU study. Effects were more pronounced. This was maybe due to minor differences in the macrophyte cover between the pond systems of the combined and the single pesticide studies. Impacts started at level 3, i.e. when the macrophytes were affected (see 5.4). In the IPU study, this level of the herbicide was the NOEL and the macrophytes were negatively affected in IPU4 and IPU5 only.

5.4 Macrophytes

Macrophytes were present in the test system. The stock consisted of *Myriophyllum spicatum*, *Potamogeton lucens*, and *P. natans*. Other species were not observed. In the single pesticide approaches additionally *Elodea canadensis* was present. Because the latter species was of minor importance, structural differences between the combined approach and the single substance studies due to this species are not expected to cause any effects.

The impact of the combined treatment is depicted in Figure 86. The covered area is presented in greater detail in Table 49. Distinct treatment effects start in level 3. The controls and level 2 had a minor decline in the covered area between day 0 and day 82 a.t.. Level 1 steadily increases. Generally, much growth took place between day 82 a.t. and day 362 a.t.. Level 3 could fully compensate the loss due to the treatment. The higher treated enclosures did not reach the initially coverage again.

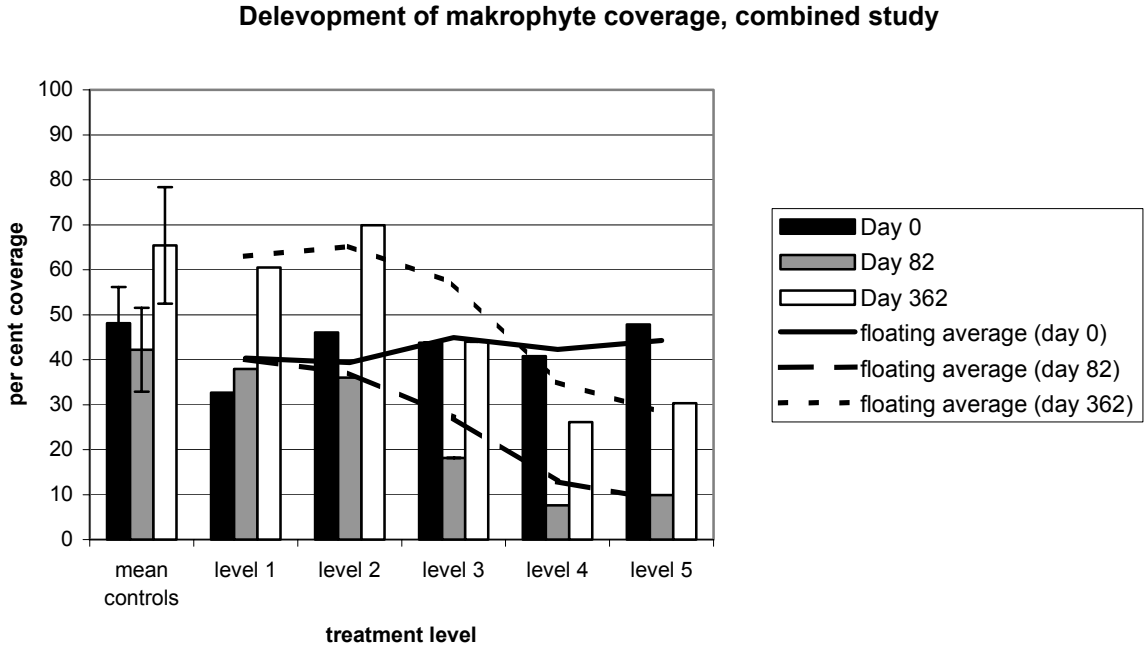


Figure 86: Development of the macrophytes in the combined study

The NOEC is level 2 containing 16 µg/L IPU. In the IPU study, it was IPU3 with 64 µg/L IPU.

Table 49: Means of the macrophyte coverage [% of the enclosure area] in the combined study

enclosure	day 0	day 82	day 362
controls	48.1	42.2	65.4
level 1	32.7	37.9	60.5
level 2	46.0	36.0	69.9
level 3	43.8	18.1	44.0
level 4	40.7	7.6	26.1
level 5	47.9	9.9	30.3

As already noted in the water quality parameters, there were certain differences between level 1 and 2. First of all, no *Potamogeton natans* was found in level 2. Second, level 2 did show a decline up to day 82 (alike the controls), whereas level 1 had an increase in the plant covered area. These differences may seem to be of minor importance, but there was a distinct difference in the pH of the two groups of enclosures (5.3.5): values were lower in level 1 right from the start of the study. Other water quality parameters showed equivalent relations between the two treatment groups (5.3). The NOEC of the macrophytes is not affected by these findings, though.

The increasing area in level 1 disagrees with the interpretation that less photosynthesis in these enclosures caused the lower pH. Another idea may be that the different plant species found in the systems may have had an influence on the periphyton and phytoplankton. Differences here could have lead to the deviations. Possibly less of the sessile flora could have

introduced the reduced pH[28], but there were no investigations of this parameter to prove this. Differences in the free floating algae did not exist in the beginning (cf. 5.5).

Summarizing, macrophyte stock may have influenced the ratio of some water quality parameters between level 1 and 2 (at least secondarily), but a definite rationale cannot be given.

For the macrophytes, all data support the NOEC of level 2.

5.5 Phytoplankton

5.5.1 Composition of phytoplankton

The phytoplankton was dominated once again by Cryptophyceae *C. erosa et ovata* and *Ch. acuta* (Table 50). These taxa alone make up over 50% of all algae. Their values are almost identical to those of the single substance studies. Only three of the taxa in Table 50 are not in the "top ten" lists of the other parts of this work (namely *Chromulina cf. ovaloides*, *Anabaena sp*., and *Kirchneriella obesa*). Ecotoxicological endpoints are therefore fully comparable between the three application scenarios.

The phytoplankton biocoenosis was composed of the classes Bacillariophyceae (13 taxa), Chlorophyceae (62 taxa), Chrysophyceae (17 taxa), Conjugatophyceae (7 taxa), Cryptophyceae (4 taxa), Cyanophyceae (10 taxa), Dinophyceae (3 taxa), Euglenophyceae (6 taxa), and Xantophyceae (2 taxa).

Table 50: Dominant species in the phytoplankton (combined approach)

	species	dominance (IPU)
1	*Cryptomonas erosa/ovata* (Cryptophyceae)	29.6
2	*Chroomonas acuta* (Cryptophyceae)	23.5
3	*Monosiga varians* (Chrysophyceae)	6.0
4	*Katablepharis ovalis* (Cryptophyceae)	5.8
5	*Chromulina* cf. *ovaloides* (Chrysophyceae)	3.3
6	*Anabaena sp*. (Cyanophyceae)	2.8
7	*Kirchneriella obesa* (Chlorophyceae)	2.4
8	*Desmarella monilifomis* (Chrysophyceae)	2.2
9	*Nephroselmis olivacea* (Chlorophyceae)	2.0
10	Cyanophyceae ssp.	1.9

The development of the distribution of the phytoplankton classes in the controls is presented in Figure 87. Only minor fluctuations can be seen in the first year. Cryptophyceae are most important all of the time; Cyanophyceae, Chlorophyceae and Chrysophyceae are present in almost constant and equivalent ratios.

[28] Indeed, the submersed structure of *P. natans* is totally different from *P. lucens*. The latter one has all its leaves under the water surface, whereas *P. natans* has floating leaves. The area where periphyton can grow may therefore be smaller in level 1.

In spring 2002 Chlorophyceae dominate the phytoplankton. Losses in Cryptophyceae facilitate this development. By June 2002 the ratios are more akin to the ones of the first year again. Chlorophyceae and Cyanophyceae are more important than in the first year, too. Their percentages show only minor variations.

In short, differences between the years are much more important than those in each vegetation period.

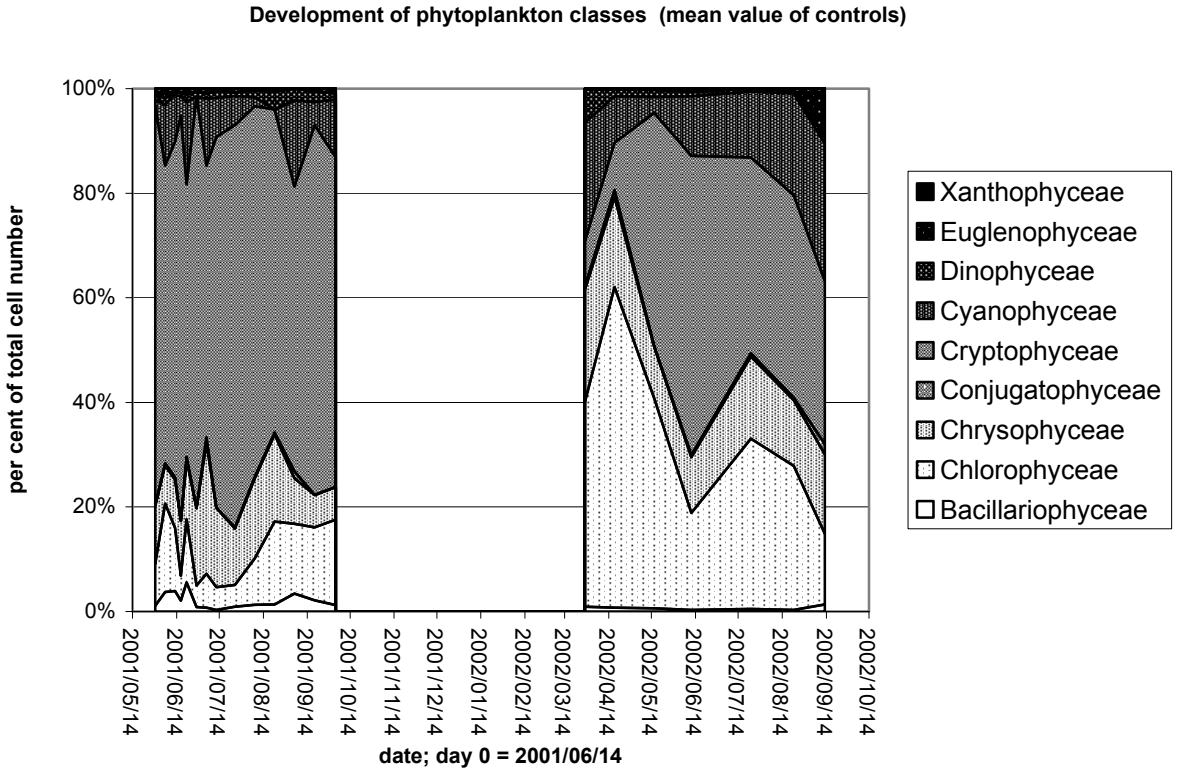


Figure 87: Phytoplankton class distribution in the combined study; controls

In contrast to the development in the controls, the class distribution is altered in the treated enclosures. Figure 88 displays the development in level 2 and Figure 89 the one in level 5, respectively.

Level 2 is distinctly differing from the controls in the year of the application only (Figure 88). The second year is similar to the controls except for the maximum in Chlorophyceae. It is lower and is reached later than in the controls. Consequently, Cryptophyceae have a higher portion of the total cell numbers. Additionally, Cyanophyceae play a slightly more important role in autumn.

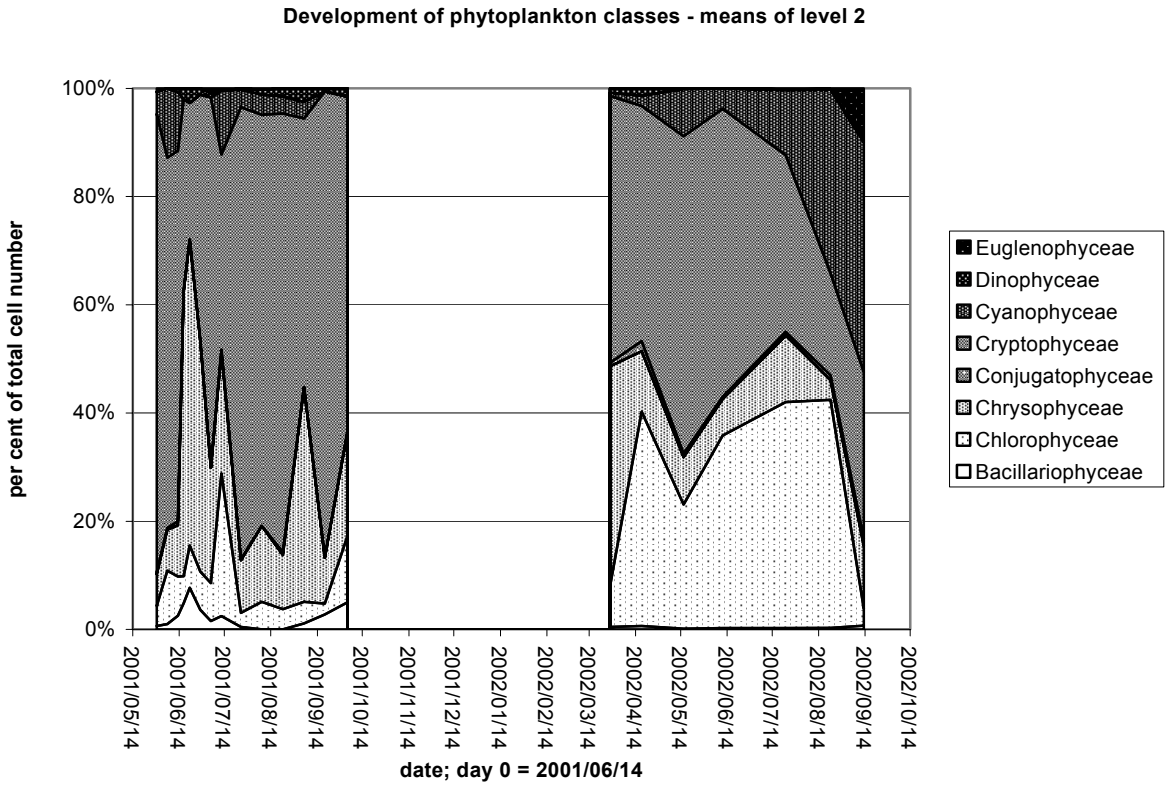


Figure 88: Phytoplankton class distribution in the combined study; level 2

The development induced by the treatment in the first year is more important. Shortly after the application, Cryptophyceae decline. By August 2001 they are in line with the controls again, but the more or less constant progression is disturbed by the Chrysophyceae. They show five peaks and their number is increased on most of the other sampling dates as well. One month a.t. Chlorophyceae have a maximum, too.

In short, the class distribution is altered to some extend in level 2. Changes apply most to Cryptophyceae and Chrysophyceae. By spring, most treatment effects are leveled out.

A more severe impact can be seen in level 5 (Figure 89). Up to one month a.t. almost no Cryptophyceae are present in the system. By the middle of August 2001 they reach control values again. In the time right after the application, Chrysophyceae and Cyanophyceae can benefit from the losses in the most dominant class. In July, Chlorophyceae become most important. Later on, the class distribution is comparable to the controls with slightly more Chrysophyceae and Chlorophyceae.

In the second year, the spring maximum is comprised of Chrysophyceae instead of Chlorophyceae. Again, Cryptophyceae are more important than in the controls (in spring). Chlorophyceae reach their seasonal maximum in summer 2002 at the expense of the Chrysophyceae. In autumn, Cyanophyceae get more and more important.

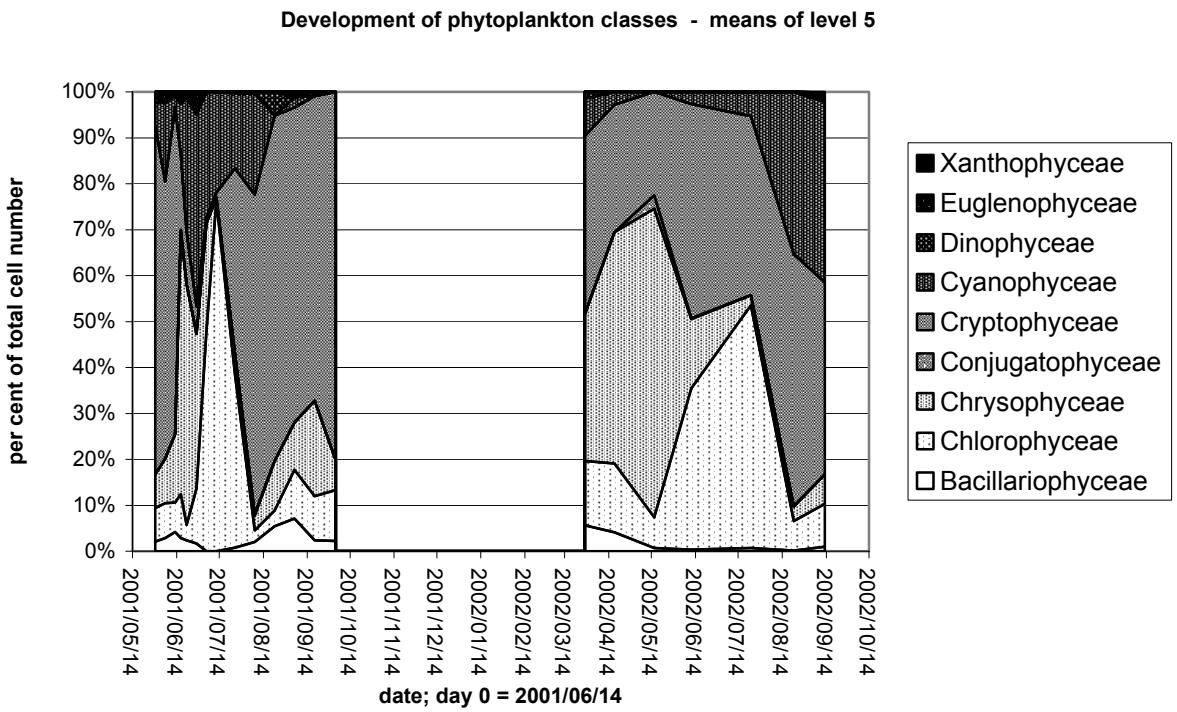


Figure 89: Phytoplankton class distribution in the combined study; level 5

In level 5, major changes of the distribution of the algae to the different classes are visible. The effects last up to autumn of the second year. As already noted before, impact on the macrophytes is shifting the structure of the ecosystem in treatment groups higher than level 2 (5.4). Recovery can only be seen when the structure (i.e. the macrophytes) gets comparable to an untreated state again.

Comparing level 5 to the IPU study (IPU5), effects in the second year are almost the same. The increase in Cyanophyceae cannot be seen. The spring maximum in Chrysophyceae is also present. The summer maximum in Chlorophyceae is not as distinct, yet still present.

Impacts in the first year also resemble each other. The major difference is that in the combined treatment no prolonged oscillating pattern can be found. Deviations of such a type are restricted to the first two months a.t.. In the IPU approach, fluctuations go on until the end of the first year with a cycle of two months. The combined application consequently affects the phytoplankton differently than the single IPU treatment.

The oscillations in the herbicide study stemmed from secondary interactions with the zooplankton that was not affected directly by the active ingredient. However, CYP treatment had direct and indirect impact on the zooplankton. As a result, the combination can be expected to show differences to the single applications. Effects and reasons will be discussed in greater detail with the abundance data of the plankton.

5.5.2 Abundance data

5.5.2a Total abundance

The development of the phytoplankton per sampling date is depicted in Figure 90. Major deviations are only found between day 14 and day 41 a.t. in treatment levels higher than number 2.

On day 14 a.t., the abundance is reduced in comparison to the controls in all treated ponds because of the treatment. On day 21 a.t., levels 3 to 5 contain more algae than the controls. This impact is even intensified on day 28 a.t., when the levels are ordered very nicely according to the kind of treatment. On day 41 a.t. merely level 4 and 5 are increased. Later on, abundance in level 5 is somewhat lower than in the controls. This effect lasts up to day 362 a.t.. The other treatment levels show only minor deviations on separate sampling days until the end of the study.

Levels 1 and 2 do not show any effects from day 21 on until the end of the study.

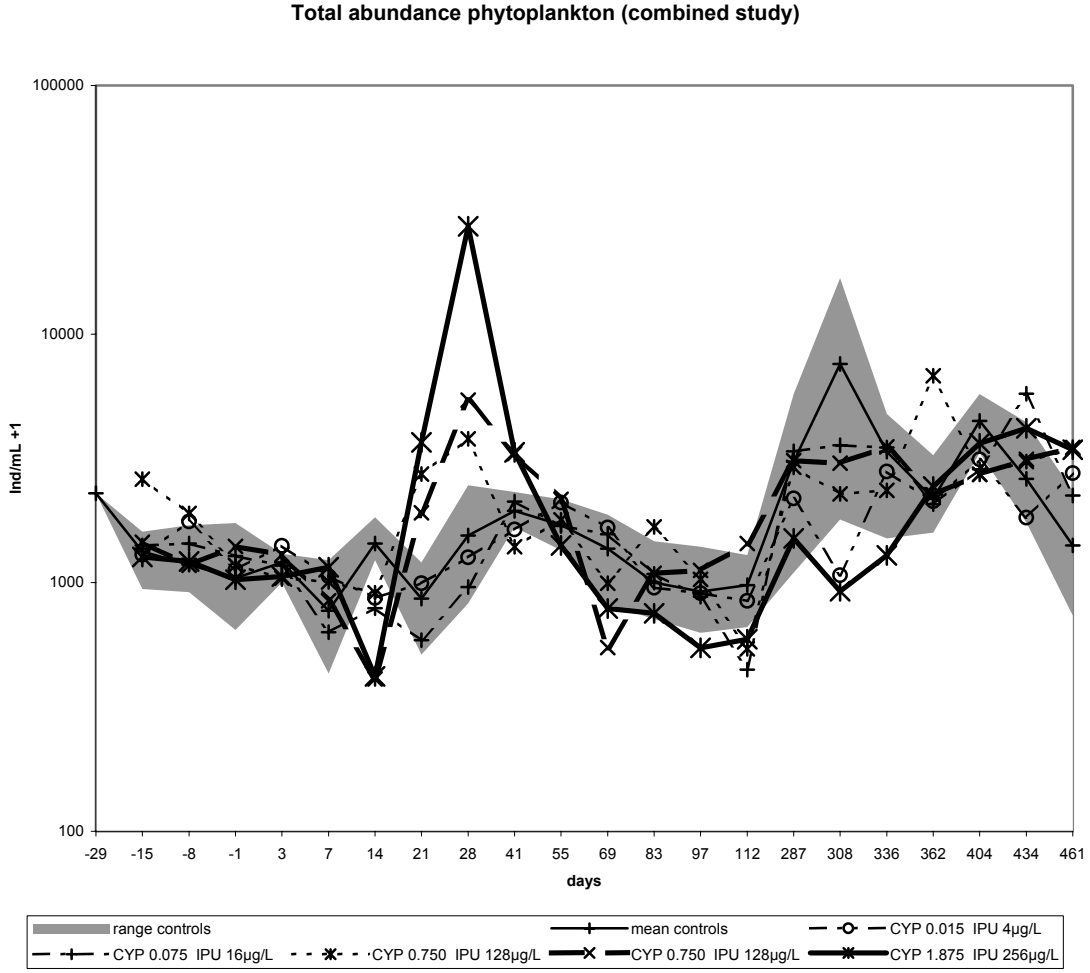


Figure 90: Development of the total abundance in the phytoplankton of the combined study

NOEC for this parameter is level 2. A NEC could be calculated for day 28 a.t. only. Values are 65.5-68.4-71.3 µg/L IPU in the combination. Both endpoints are almost the same as in the IPU study, but there is a big difference in the effects of the treatment.

With IPU alone, there was a treatment related oscillating pattern in the algae. It was traced back to the interaction of the algae with their main grazers. As already seen in the CYP study, these grazers, mainly *Simocephalus vetulus*, are affected by the insecticide. Additionally, effects on the main predator of the zooplankton were found that had a promoting effect on some zooplankton taxa.

In the combined treatment, the oscillating pattern in the phytoplankton is detectable with one fluctuation only. IPU reduces the algae right after the application. Three weeks after the application the reduced grazing pressure (due to the impact of the insecticide on the zooplankton, cf. 5.6.2a) enables the algae to grow to higher numbers. Zooplankton is not affected negatively any more in level 3 from day 28 onwards and even increased afterwards in all treatment levels, beginning in level 3 (5.6.2a). A rationale for the development in zooplankton is given there.

As a result for the algae, grazing pressure on them can be assumed to be even higher than in the controls. Abundance of the phytoplankton is only decreased to some extend in level 5. The algae seem to be able to compensate for the losses due to zooplankton grazing via a better numerical reaction. Such a development could be promoted by better nutrition (cf. WENDT-RASCH 2003), light conditions etc. for the algae due to the decay in the macrophytes (cf. 5.3 and 5.4). Herbicide dose in level 5 is high enough to cut down the abundance even under these circumstances (level 4 has the same amount of grazers but more algae).

So why are there no further oscillations in the phytoplankton of the combination? A possible interpretation is:

1. IPU reduces algae in the beginning, CYP the zooplankton → minima on the first few dates
2. Algae that are not sensitive to IPU grow to higher numbers due to reduced grazing → maximum in algae
3. Zooplankton is getting back to normal as CYP is lost from the water column → algae are reduced to normal
4. Decaying macrophytes provide better growth conditions for the algae *and* predation on zooplankton is still lowered → higher zooplankton abundance (grazers) controls the better growing algae (except for level 5, where enough IPU is still present to keep the phytoplankton slightly lowered).
5. While CYP effects on the zooplankton predators are leveled out (and thus the promotion of the grazers), IPU declines and the macrophytes recover. This development needs more time the higher the treatment level had been. In any case, no fluctuations become visible, because influence on the plankton is exerted by at least five influences which can even be opposed to each other (CYP and IPU impact, grazing and predation pressure, and the development in the macrophytes).

In this way, the additional effects of the combined treatment do not allow building up the oscillation seen with IPU treatment alone.

5.5.2b Species richness

The number of taxa found in the phytoplankton of the combined study is presented in Figure 91. No effects were visible in the year after the application.

Level 3 is over the control range between day 14 a.t. and 28 a.t., level 4 on day 21 and 28 a.t., and level 5 as well as level 1 merely on day 28 a.t.. Level 2 shows only negligible deflections. Level 5 has slightly less taxa between day 55 and 112 a.t..

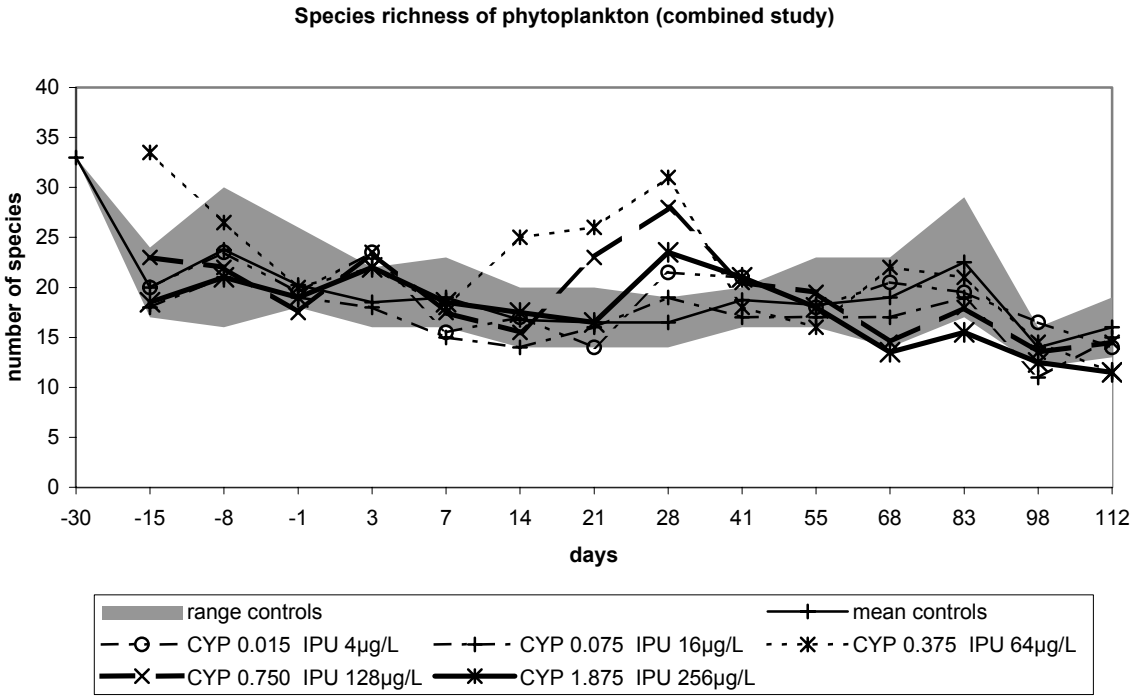


Figure 91: Species richness (taxa) in the phytoplankton of the combined approach

NEC values calculated for both pesticides are listed in Table 51. For the IPU content, values are higher than in the IPU single substance study. The NEC there was about 50 µg/L a.i., while it is about 300 µg/L here. The duration of the impact is different, too. The IPU study needed about 97 days to recover; in the combination, no more effects are already visible on day 41 a.t. (excluding the lowered taxa richness in level 5). Additionally, treatment relation is different from IPU treatment alone (which was rather a decrease).

Table 51: NECs of the taxa richness in the phytoplankton of the combined study

NEC	NEC [µg/L CYP]	N	NEC [µg/L IPU]	N
lower	0.070	3	182.2	6
middle	0.256	3	259.2	6
upper	1.180	3	433.6	6

With respect to the CYP study, the impact in the combination is equally related to the treatment but with a higher NEC (about 0.050 µg/L with CYP alone). Effects with CYP alone started already on day 7 a.t. in all affected levels and lasted up to day 27 (exclusively). Here in the combined study the reaction to the treatment is identical, but the increase begins in level 3 earlier than in the other affected levels, it lasts somewhat longer, however (up to day 41 a.t.).

An explanation can be given in this way: Taxa susceptible to IPU vanish treatment related. At the same time, grazing is lowered by CYP action (cf. 5.6.2). Thus, by and by more taxa can be found in the system because this effect is enabled only by the decline in IPU residues. By the time the zooplankton reaches control levels again (day 41 a.t.) effects are leveled out.

What must be dealt with is the decrease in taxa richness in level 5. Plainly, it is related to the higher grazing in the treatment level, because numbers of zooplankton are increased after day 41 a.t.; nicely related to the treatment (Figure 98). Level 4 has almost the same abundance of zooplankton, but less IPU residues, of course (Figure 75). Consequently, the algae can better compensate for the losses. An analogous interpretation is valid for the other treatment levels.

So here we see a combination effect that is lower than the effects of the single applications as well in the NEC as in the duration (level 5 excluded).

5.5.2c Chroomonas acuta

This Cryptophyceae is one of the most important taxa in all the studies presented here. It was clearly affected by the IPU treatment with a NOEC of 16 µg/L IPU. Decreases were found up to day 41 a.t.. CYP treatment showed only minor impacts, also hinting at a slight direct toxicity.

The effects of the combined treatment are presented in Figure 92. In the second year, no impact was detectable.

All levels except level 1 were clearly affected. The curves are almost identical to the ones of the IPU study and the recovery time is comparable (no more adverse effects on day 41 a.t.). NOEC is lower, 4 µg/L IPU in the combination. NECs are 2.7-3.5-4.8 µg/L IPU in the combination (n=3) corroborating the NOEC. The NEC in the single substance approach has been about 10 µg/L IPU.

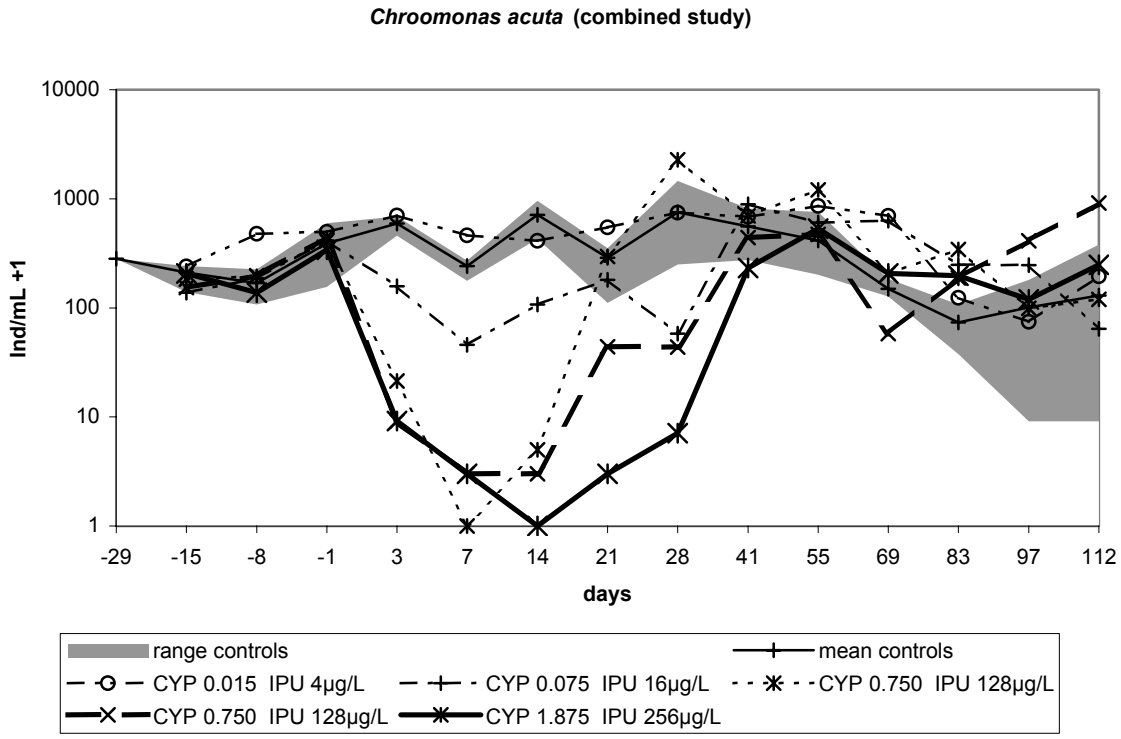


Figure 92: Development of *Chroomonas acuta* in the combined study

It can be concluded that the combined treatment had a more severe impact on *Chroomonas acuta* than the single substances. Their impacts seem to add up.

5.5.2d Cryptomonas erosa et ovata

This taxon has been the most important one in the IPU study and in the combined approach. It is closely linked to the zooplankton because it is one of its main food resources (cf. the IPU and the CYP study).

In the CYP study, decreases were found mainly in the second year. NOEC was 0.015 µg/L CYP.

In the IPU study, the *Cryptomonas ssp.* were sensitive towards the herbicide with a NOEC of 16 µg/L IPU. After a fortnight they entered a fluctuating pattern that was related to the toxin, the zooplankton and the macrophytes. In the second year, over-compensation for the losses was seen in the enclosures treated higher than the NOEC from June on.

The development in the combined approach can be seen in Figure 93. Up to day 28 a.t. the herbicide impact is very much evident. NOEC is 16 µg/L IPU in the combination (level 2). The impact is more pronounced, because possibly the IPU action is promoted by the minor toxicity of CYP (3.6.2d). Additionally, the combined treatment seems to have altered the linkage between the algae and the zooplankton. This can be the reason why the oscillations are not able to build up (as discussed earlier). The NEC values are 1.8-6.5-29.1 µg/L IPU in the combination (n=4) backing the NOEC.

Later on in the first year, numbers stay around the lower end of the control range, because in this time slot zooplankton grazing is increased (5.6.2a).

In the second year, the lowered abundance is still due to increased grazing; a development that is triggered by the insecticide in the same way as in the single substance approach: Less predation (5.6.2c) leads to more grazers (5.6.2a) and therefore less algae.

In autumn the decreases to the controls are over-compensated. The point of time quite nicely relates to the grazers getting back to the control range (5.6.2a). This has not been seen in the CYP study, but a comparable development was found in the IPU approach. So this effects is triggered by the herbicide. As in the single substances study, the intensity of the effect is related to the impact the macrophytes had received due to the treatment.

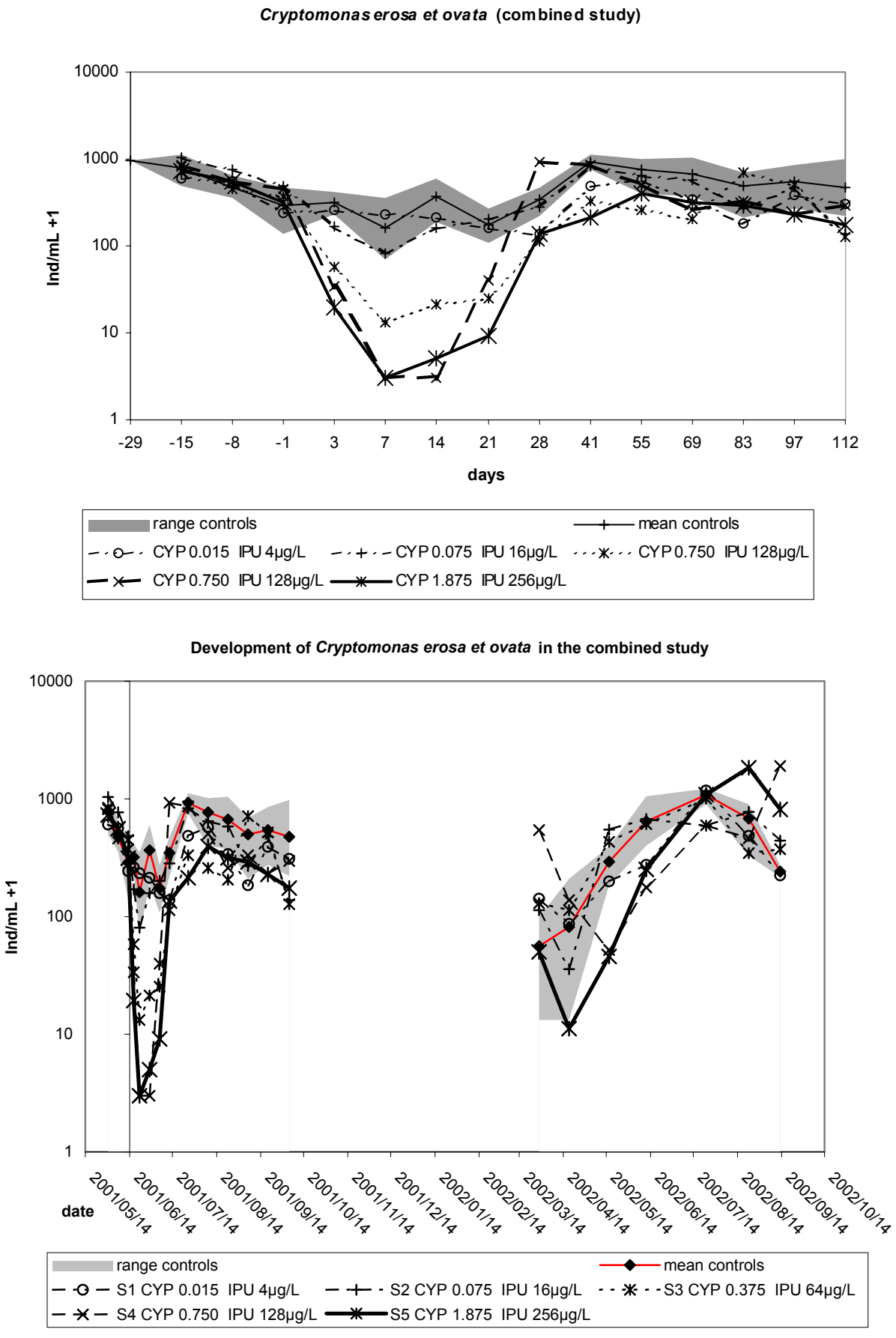


Figure 93: Development of *Cryptomonas ssp.* in the combined study; top: year one, bottom: both years

In short, the impact both pesticides had in the single application could be seen to some extend in the combination. Effects in the first year were even more pronounced. NOECs are identical, though.

5.5.2e Bacillariophyceae

Silicate amounts indicate an impact of the combination on the diatoms (5.3.8b). In the single substance applications, numbers were altered to some extend. With CYP, minor increases were seen around day 55 a.t.. With IPU, effects were not too pronounced either. A decrease starting around concentrations of 64µg/L herbicide (IPU3) was indicated. Increases in the silicate contents were attributed to a possible impact on the periphyton.

In the combined treatment, effects are not clear either. Numbers, even in the controls, are fairly low, most of the time around 30/L. Level 3 has the highest abundances over the control range on day 21 and 28 a.t.. On day 28 a.t., level 1 and 2 are next over the control range, followed by level 4. On day 41 a.t. level 4 is increased; on day 55 level 5 additionally as well. Day 69 a.t. exhibits abundances higher than the controls in all but level 2. A treatment relation cannot be seen, though.

Effects even in the combined treatment are quite as unclear as in the single substance studies. NEC calculations were possible on three dates, but their explanatory power is rather low. They are 40-118-(642) µg/L IPU in the combination and thus higher than with IPU alone. Further interpretation is not advisable.

In short, the two pesticides used do not exert a major impact on the planktonic Bacillariophyceae even in their combination.

5.5.2f Chlorophyceae

The progression of this class of algae is depicted in Figure 94. They have a sharp, treatment related peak between day 14 and 41 (01/06/28 to 01/07/25), are then lowered to some extend until June 2002, and mostly inside the control range up to the end of the study. NOEC is level 2, NECs are 93.0-93.9-113.9 µg/L IPU in the combination (n=4) (0.255-0.290-0.517 µg/L CYP, respectively).

The pattern found here resembles the one of the taxa richness (5.5.2b). Explanation for the development can be looked up there. Chlorophyceae seem to profit most from the combined treatment, until grazing gets higher.

Compared to the single substance studies, this development could be expected. With IPU, minor decreases were found in IPU4 and IPU5. Secondary effects were hinted at, too. With CYP, the peak right after the application was also seen, but it was already present on day 7 a.t.. NOEC for CYP was 0.015 µg/L a.i.. In the combination, the NOEC is higher and the peak occurred later. This is due to the herbicide action that inhibits the increase (secondary effect of CYP!). The later decrease is due to more grazing pressure of the zooplankton. Increases there (5.6.2a) have been higher in the combination than with CYP alone, where such a decrease in Chlorophyceae could not be found.

With minor exceptions of level 2, long lasting effects were only visible in concentration levels above the NOEC.

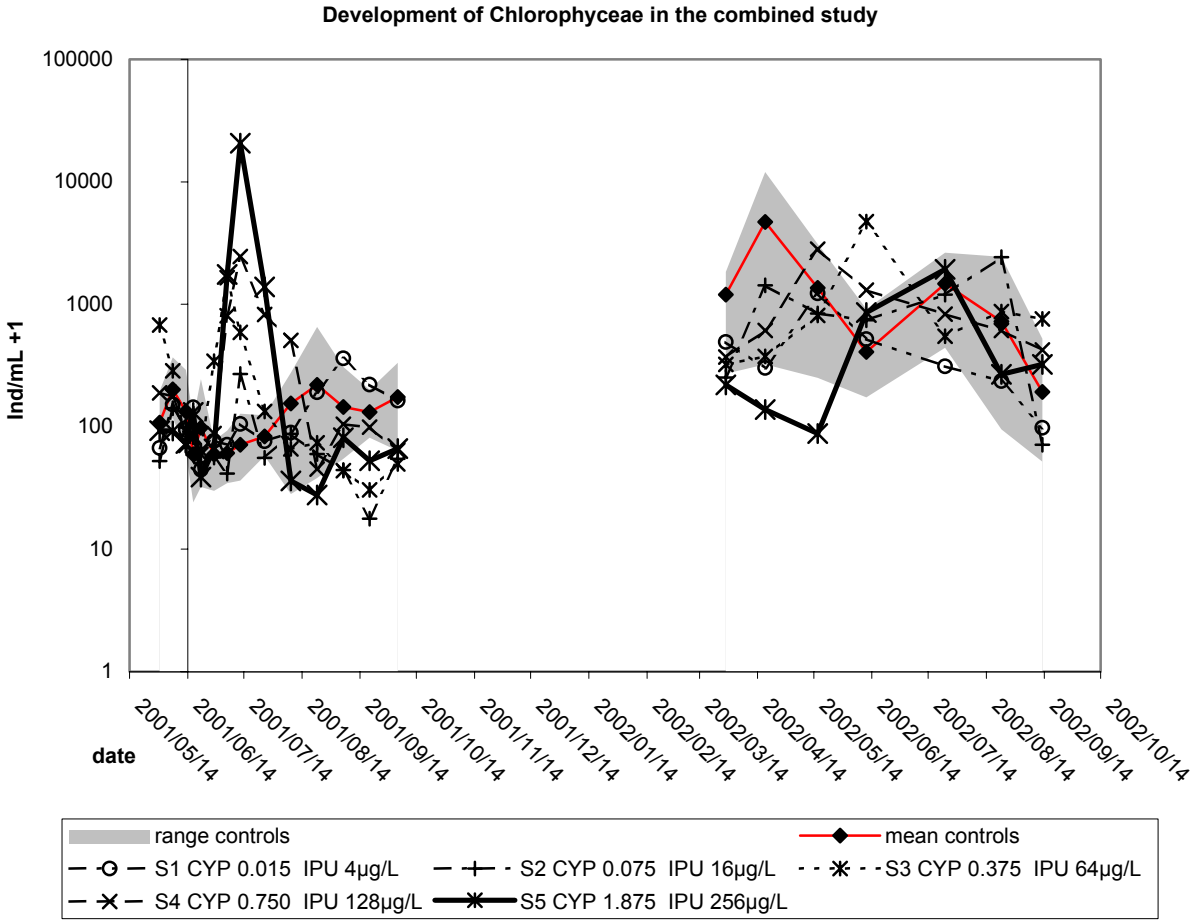


Figure 94: Development of the Chlorophyceae in the combined study

In short, the combined action lead to a higher NOEC that can easily be explained by the effects of the single substances.

5.5.2g Nephroselmis olivacea

This species had been very sensitive towards IPU (NOEC 4 µg/L), but was not affected by CYP. Here in the combination, no effects were observed in both study years. No NOEC or NEC could be calculated. The IPU impact must therefore be totally leveled out by the (direct or indirect) CYP action on the grazers. In the IPU part, this taxon was discussed as being able to show a quick numerical reaction ("r" strategy). This ability can enhance the finding that IPU in the combination is not able the cut down *Nephroselmis olivacea* in numbers. With CYP alone, no increase in the abundance was visible, although the insecticide reduced the number of grazers. Such a development might be explained by the fact that, with CYP alone, no change in the nutrition of the algae was achieved. Such was the seen in the combination (5.3) and a growth of the algae is thus facilitated. As a result, IPU action was not visible.

The combination had again less impact than at least one of the pesticides alone (IPU in this case).

5.5.2h Chrysophyceae

This class has been important when interpreting some of the water quality parameters of the combined treatment study (5.3.9). In Figure 95 the development of these algae is presented. They show a major increase in all but level 1 on day 3 and day 7 a.t.. Abundance in level 2 and 3 is still above the control range on day 14 a.t.. On day 21 a.t. level 4 and 5 are increased. Later on in 2001 only minor alterations are found.

In 2002, level 5 is lower than the controls in April, July, and August, and higher in May. The other treatment levels are most of the time in or slightly lower than the control range.

NOEC is level 1; NECs are higher: 37.8-46.8-71.7 µg/L IPU in the combination (n=4).

As noted before, this class proved to be influenced rather strongly (and differently to the single substance approaches) by the combined treatment. Not only the percentages of the total number of algae was increased by the treatment (Figure 87, Figure 88, Figure 89), but also the abundance itself.

The reaction in the combination resembles the one found in the CYP study: an increase right after the application (due to reduced grazing). The NOEC is lower in the combination, level 1 instead of CYP2. With CYP alone, in CYP5 the increase was smaller; possibly due to a minor direct toxicity of the insecticide. This finding cannot be seen in the combination. Additionally, no decrease is seen after a month a.t. as with CYP alone. As noted above, grazing in the combination study is higher from this point of time on (alike in the CYP study). The decrease is therefore more likely to occur. IPU action may lead to the different development. The reaction to IPU alone was rather unspecific and more of an secondary nature even in the first year. The only clear effect had been an increase in the enclosures higher than IPU2 (=NOEC) in spring of the second year. In the combination, IPU seems to alter competition outcome in such a way that the minor direct action of CYP is masked and that no decrease due to more grazing can be seen. This is most possibly due to the reduction of the sensitive, but highly abundant taxa *Chroomonas acuta* and the *Cryptomonas ssp*. (5.5.2c and 5.5.2d), additionally assisted by better nutrition (5.3.8). The increase of the Chlorophyceae in the second year (IPU study) is not visible in the combination. This can be explained by the higher grazing pressure due to the CYP action on the zooplankton (5.6.2a) which is able to cut down the algae. Such an increase in zooplankton could not be seen with IPU alone.

The slightly lowered abundances in the course of the second year can be addressed to the general development of the pond systems. Macrophytes become more and more important in the enclosures (5.4) when the herbicide is degraded, thus the algae may be affected negatively due to competition reasons. The reaction is too unspecific, however, to be interpreted in further detail.

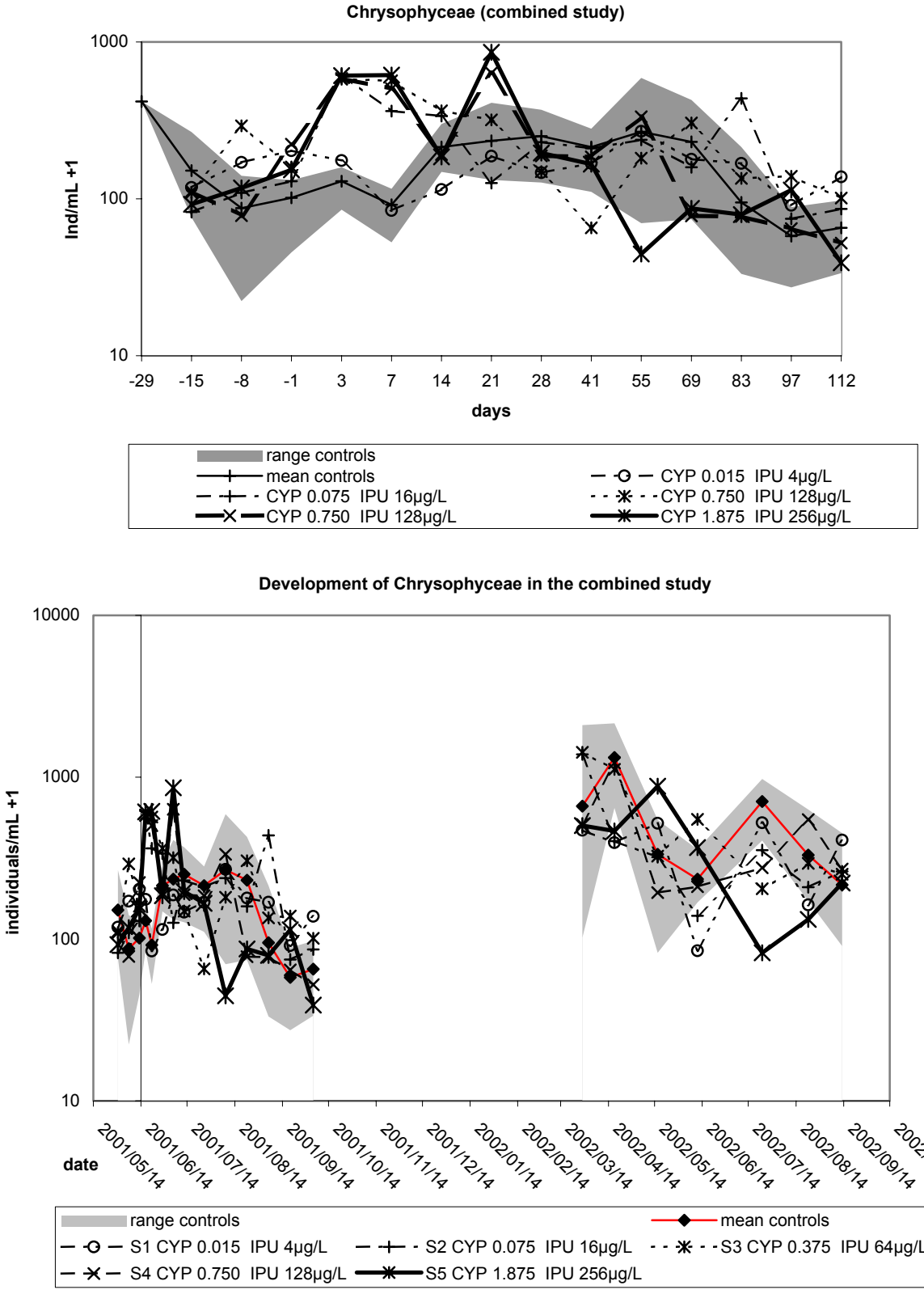


Figure 95: Development of the Chrysophyceae in the combined study; top: year one, bottom: both years

To sum it up, Chrysophyceae are influenced more intensely and differently than in the single substance studies. Secondary interactions can explain the development of these algae as the pesticides are degraded.

5.5.2i Other algae

Here a short survey of algae that proved to be sensitive to the combined treatment is given. These taxa were not influenced as much in the single substance studies, so a combination effect can be assumed. Since the taxa increase shortly after the application, they may well take advantage of the reduced grazing and lowered abundances of the main algae (see above). Attributing these combined effects to special "combination" reasons is not advisable, because data of the single substance studies cannot generally be interpreted as far as necessary.

The Cyanophyceae increased between day 14 and 69 a.t. in level 3 to 5. NOEC is level 3, corroborated by the NECs of 9.0-25.4-97.8g/L IPU in the combination (n=3). Increases in level 3 are thus not significant.

Katablepharis ovalis (a Cryptophyceae) increased on day 3 a.t. in level 3 to 5 and again on days 28 to 41 a.t.. NEC is 3.1-6.4-13.1 µg/L IPU in the combination (n=2) backing the levels where observations related to the treatment were made.

Kirchneriella obesa is the Chlorophyceae that was able to profit most from the combined treatment. Apparently it is not too sensitive towards IPU because it only increases in the higher treated levels (Table 52); data is given for selected dates when particularly many algae were found. Enclosures that are not listed contained no individuals at all during the whole study.

Table 52: Abundance [Ind/mL] of *Kirchneriella obesa* in the combined study

enclosure	day 21	day 28	day 41
K1 0µg/L	0	0	0
K3 0µg/L	0	0	90.8
K4 0µg/L	0	0	0
S1a CYP 0,015 IPU 4µg/L	0	0	0
S3 CYP 0,375 IPU 64µg/L	0	0	13.2
S3a CYP 0,375 IPU 64µg/L	0	0	9.1
S4 CYP 0,750 IPU 128µg/L	943.9	3568.6	1201.1
S4a CYP 0,750 IPU 128µg/L	21.4	421.4	0
S5 CYP 1,875 IPU 256µg/L	560.2	37571.7	2266.5
S5a CYP 1,875 IPU 256µg/L	2837.9	1152.1	74.4

This short-term increase was high enough for *K. obsea* to become one of the most dominant species (Table 50).

5.5.3 Community analysis

5.5.3a Shannon index and evenness

These two indices showed no clear effects. The curve of level 5 is comparable to the one of the total abundance. No sensible NEC could be calculated, all values were much higher than the concentrations that were used in the pond system.

H_{max} is 3.7, the mean for the evenness is 0.6 and 1.8 for the Shannon index, respectively. These values are comparable to the single substance studies and in this way indicate that the finding between the different approaches can be compared.

The evenness had no effect levels of about IPU2 and CYP1 with the pesticides introduced separately. In the combination, effects seem to be leveled out. A general interpretation is that an insecticide allows more algae to grow (via grazing!) (cf. for example BROCK, VAN DEN BOGAERT *et al.* 1992, HANAZATO and KASAI 1995, HAVENS 1995, VAN DONK, PRINS *et al.* 1995, BOYLE, FAIRCHILD *et al.* 1996, RAND, CLARK and HOLMES 2001, VAN DEN BRINK, HARTGERS *et al.* 2002) and a herbicide decreases their abundance and/or their taxa richness (trivial). Consequently, the combination may not exert any influence.

Findings in the abundance data are opposed to this interpretation. Especially the findings in the total phytoplankton abundance and the taxa richness (5.5.2a and 5.5.2b) rather indicate that the indices used here are not capable of integrating the community in such a way as to preserve the treatment related deviations. Restricting analyses to them will lead to a misinterpretation of the treatment effects.

5.5.3b RAD index

The RAD index is an integrated measure that proved to be sensitive enough to allow the detection of alterations in the community structure. For the combined treatment, it is displayed in Figure 96. NEC values calculated for both pesticides are presented in Table 53. They are suggesting significant effects at concentrations higher than level 1.

Major deviation that are related to the treatment intensity are found between days 3 and 41 a.t.. On days 21 and 28 a.t. all treated enclosures are above the control range; on the other days effects start in level 2. After day 41 the treatment relation is loosened. Please note that only levels 1 and 2 are within the control range for some occasions up to day 404 a.t.. On the last two sampling dates effects of the treatment are more or less leveled out.

NECs of the single substance studies were approximately 0.3 µg/L CYP and about 40 µg/L IPU. CYP treatment alone was responsible for deviations for about three months and later on after about one year a.t. (recovery of *Chaoborus crystallinus* and thus altered grazing pressure in CYP3-5). IPU application effects in the lower treated ponds were balanced after three months as well; impact in IPU4 and IPU5 were preserved to some extent until the end of the study.

The diagram shows that the impact of the combined treatment affected the phytoplankton seriously. All NECs are lower than in the single substances approaches. Recovery is seen after more than one year a.t. and the differences to the controls are more pronounced that in the single application approaches.

Table 53: NECs for the RAD of the phytoplankton in the combined study

NEC	CYP [µg/L]		IPU [µg/L]	N
upper	0.029	6	7.48	6
middle	0.021	6	5.55	6
lower	0.015	6	4.20	6

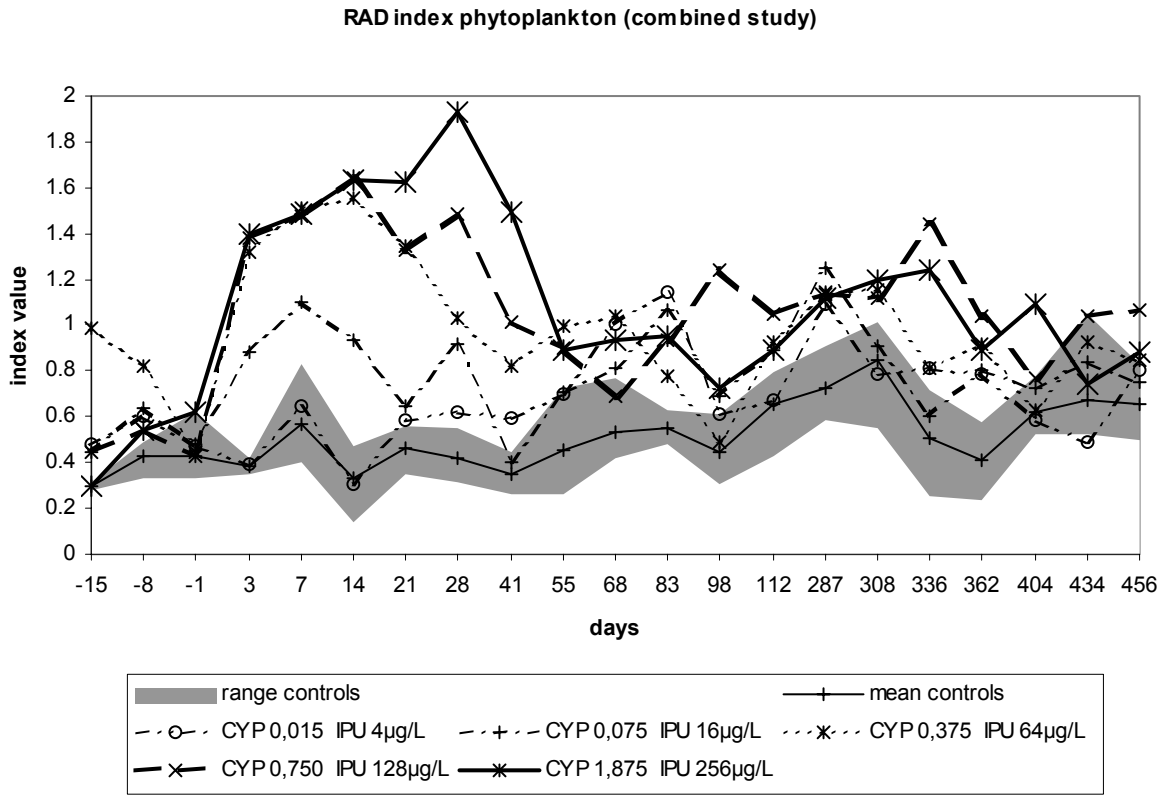


Figure 96: RAD index of the phytoplankton in the combined approach

In summary, the combined application altered the phytoplankton more thoroughly than the single ones.

5.5.3c PRC analysis

The outcome of the PRC analysis is depicted in Figure 97. Secondary interactions were discussed as the reason for some of the changes found in the phytoplankton (5.5.2). In the single substance approaches, the multivariate analysis was not able to resolve such effects because they were opposed to the treatment level, especially with the insecticide. $NOECs_{community}$ were 16µg/L IPU and 0.075 µg/L CYP, respectively; i.e. the second treatment level.

In the combined treatment, some interaction eliminate each other (reduced grazing – herbicide toxicity, cf. 5.5.2g). Additionally, the linkage between phyto- and zooplankton is modified (no oscillations as with IPU alone, cf. 4.6.2a and 4.6.2d). PRC analysis might therefore be able to give convincing results. This is indeed observed (Figure 97).

A distinct treatment relation is found until day 55 a.t. when PRC indicates recovery. Levels 1 and 2 seem to be unaffected. The more extensive deviations are seen on day 28 a.t..

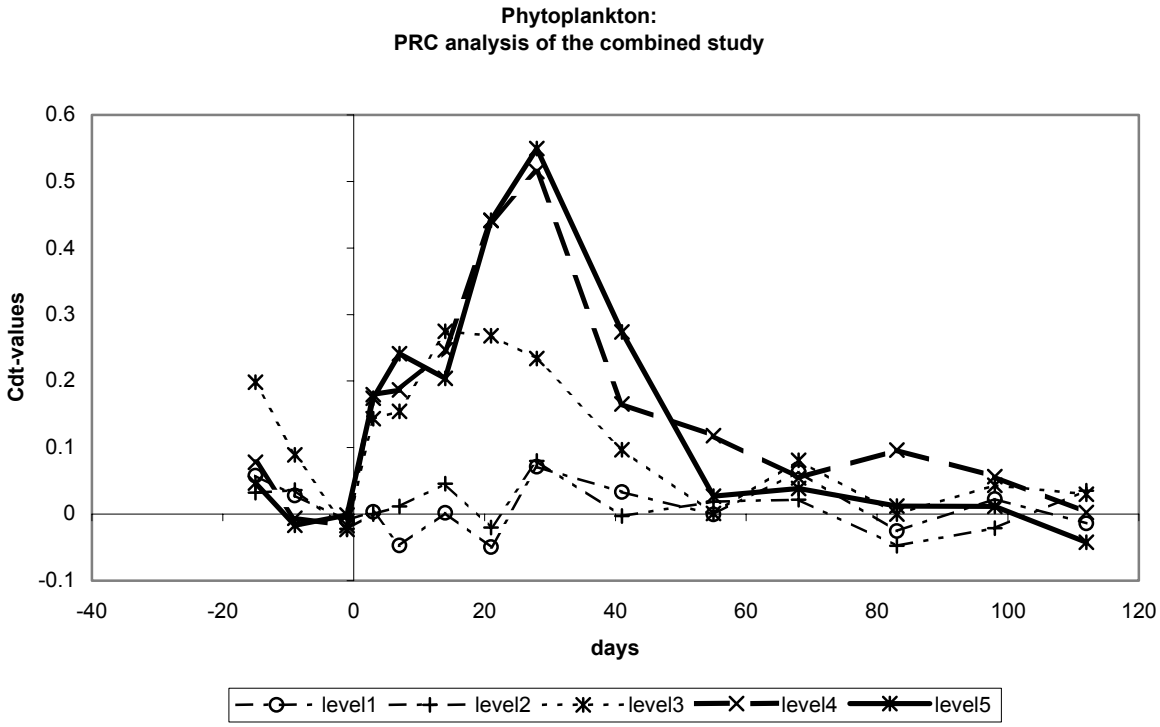


Figure 97: PRC diagram of the phytoplankton in the combined study

This analysis is significant, p=0.005. It explains 40.7% of the variances by the treatment of which 19.3% are displayed. 20.1% of the variations are explained by the sampling day. Table 54 lists the relevant taxa in this analysis.

Table 54: Important phytoplankton taxa in the PRC analysis on data of the combined study

taxon	score
Kirchneriella obesa (Chlorophyceae)	1.3185
Cyanophyceae sp.	1.0166
Anabaena sp. (Cyanophyceae)	0.8442
Chromulina sphaeridia (Chrysophyceae)	0.7230
Scenedesmus cf. *tenuispina* (Chlorophyceae)	0.7216
Gomphosphearioideae sp. (Cyanophyceae)	0.5863
Coenocystis subcylindrica (Chlorophyceae)	0.5490
Cryptomonas erosa/ovata (Cryptophyceae)	-0.6999
Chroomonas acuta (Cryptophyceae)	-1.1628

The algae referred to in this list, at least their class, were all discussed in greater detail with the abundance data (5.5.2). Both those were increased by the treatment and the negatively affected ones were found. Effects on the increasing taxa are more pronounced than in the single substance approaches, a fact that is reflected by the higher number of taxa that take advantage of the treatment (7 taxa in the combination, with IPU 2 taxa, and with CYP 5 taxa[29]).

[29] It is an amusing fact that simply adding up the number of influenced taxa equals the number of the affected ones in the combined approach. However, this is pure coincidence!

$NOEC_{community}$ is level 2, corroborated by the NECs: 11.4-19.0-19.3 µg/L IPU in the combination.

Comparing the NOECs with each other, the combined treatment does not affect the algae more severely than the single substances. Please note that the effects in CYP alone were significant for a shorter period of time (PRC for the whole first year was not significant) and that the PRC curves are better treatment related than with IPU. Effects may thus start at the same concentration as with the single substances, but if there is an effect, it is more pronounced. In this way, the combination does influence the community stronger.

5.5.4 Overview of treatment effects of the combined application on phytoplankton

The combined treatment did not alter the phytoplankton in an uniform way compared to the single substance studies. Neither a singularly positive effect[30] nor a negative one was observed. NOECs/NECs are not altered severely most of the time, either (Table 55). Endpoints had to be scrutinized to lead to an interpretation.

Table 55: Summary of NOEC data of phytoplankton parameters (combined study)

taxon	NOEC [µg/L]	direction of pesticide influence on data /remarks
Chlorophyceae	0.075/16 µg/L	peak in the first month, then lowered
Chroomonas acuta (Cryptophyceae)	0.015/4 µg/L	decrease, additive action
Chrysophyceae	0.015/4 µg/L	combination effects
Cryptomonas erosa et ovata (Cryptophyceae)	0.075/16 µg/L	no oscillating pattern, combination effects
Cyanophyceae	0.375/64 µg/L	increase
Desmarella moniliformis (Chrysophyceae)	n.n.	
Monosiga varians (Chrysophyceae)	n.n.	
Nephroselmis olivacea (Chlorophyceae)	n.n.	very sensitive to IPU alone, thus combination effect
total abundance	0.075/16 µg/L	slight decrease, then peak in the first month, secondarily decreased
$NOEC_{community}$	0.075/16 µg/L	effects only up to day 55 a.t.

The total abundance is lowered to some extend on day 14 a.t. (IPU action) and increased later (up to day 41 a.t., secondary CYP action). Up to the end of the study, total abundance is a little bit lower because the grazing pressure from the zooplankton is higher (5.6.2a). Such a decrease due to more grazing was not seen with CYP alone, although the abundance of some grazers was increased, too. The most prominent deviation from the IPU study is the absence of a fluctuating pattern. CYP action thus alters the linkage between the phyto- and the

[30] Such an effect could be caused by the insecticide reducing grazing pressure while the herbicide action is identical to the single substance approach.

zooplankton when combined with the herbicide. The no effect levels of the combination and IPU alone are comparable.

The number of taxa increased treatment related between day 14 and 41 a.t. (secondary CYP action). From day 55 onwards, marginally fewer taxa were found than in the controls. This parameter is less sensitive than in the single substance studies.

Chroomonas acuta was sensitive towards IPU and hinted at a minor sensitivity towards CYP. In the combination, an additive effect was seen for the NOEC. It is level 1 instead of treatment level 2 of the single substance approaches. Whether CYP is really somewhat toxic for this species cannot be clarified. The higher sensitivity may also stem from the outcome of competition between the algae themselves, because some of them, especially Cyanophyceae, Chrysophyceae and Chlorophyceae, were able to take advantage of the combined treatment.

The Chlorophyceae showed the same development as the taxa richness but with a sharper peak early after the application. The NOEC is higher than with CYP alone, but lower than with IPU. Some taxa of the Chlorophyceae were also found important for the increase in phytoplankton by the PRC analysis.

The Chrysophyceae were able to take advantage of the CYP treatment. In the combination with IPU, the increase was higher (the NOEC consequently level 1 instead of CYP2) and the hinted at minor direct toxic action of the insecticide could not be seen. Secondary interactions eliminated effects that were seen in the single substance studies in the course of the time after about two months.

The Cyanophyceae as well as some other taxa played a certain part in the increase in phytoplankton about three weeks a.t.. Their importance is corroborated by their species scores in the PRC.

The *Cryptomonas ssp*. were quite susceptible to the combined treatment. This effect was more obvious than with IPU alone, because no oscillations were found. An explanation can be given in the same way as with the total abundance. After day 41 a.t., when grazing pressure was higher (5.6.2a), abundances were a little decreased. This finding corroborates the interpretation given in the other parts of the study that *Cryptomonas* is the main nutrition for the zooplankton. Other algae were not reduced due to the increase in grazers. NOEC for *Cryptomonas* is identical to the IPU study (level 2), but higher than with CYP (0.015 µg/L, level 1). This finding, together with the absent oscillations, indicates the changed relation between algae and grazers due to the combined treatment.

Nephroselmis olivacea was very sensitive to IPU (NOEC=4 µg/L). In the combined application, no effects at all were observed. Reduced grazing (CYP action) thus totally outweighed the herbicide action.

Shannon index and evenness do not indicate any changes due to the combined treatment. Since there are obviously some, these measures are not very applicative when investigating more complex ecotoxicological data.

The no effect level of the RAD is level 1 (lower than merely with CYP or IPU). Compared to the single applications, the combination affected the phytoplankton more severely, especially in the first two months a.t.. Recovery took as long as with IPU alone (about one year) but deviations from the control range were kept higher for a longer time.

PRC results in the same $NOEC_{community}$, but the effects were more pronounced. Recovery was seen after about two months, when the zooplankton decline had recovered.

To sum it up, effects that were triggered by CYP action (direct or indirect) were more pronounced but sometimes a little bit delayed due to the IPU impact. These early effects were limited to about two months a.t., when CYP is completely lost from the water column and early secondary effects have been balanced. Please remember the generation time of many plankton organisms that is between a few days and three months. This is exactly the time it took for the impact to be eliminated and corresponded well to the zooplankton total abundance (5.6.2a). Later secondary effects - as seen many time in both the CYP and the IPU study - were balanced out so that the combined impact was smaller. The only parameter indicating longer lasting major effects is the RAD. The top-down control of the zooplankton was thus more important than the bottom-up control of the algae for phytoplankton development in the combined treatment.

It is concluded that impact of the combined treatment started in level 2 (NOEC level 1): *Chroomonas acuta*, the RAD, and the Chrysophyceae strongly suggest this value. This is less than with the herbicide alone.

Recovery in phytoplankton took place faster than with the herbicide alone for some parameters. It cannot be concluded that a combined insecticide-herbicide impact has generally a shorter but more severe impact on the algae. The fast recovery seen here is the outcome of competition in the test system used and may well be totally different in a system that is not dominated by macrophytes as much (cf. for example BROCK, VAN DEN BOGAERT *et al.* 1992).

5.6 Zooplankton

5.6.1 Composition of zooplankton

The zooplankton community was divided in 37 taxa: 10 taxa of Cladocera, 3 of Copepoda (including Nauplius larvae), Ostracods, 22 Rotifers and one insect larvae (Diptera), *Chaoborus crystallinus*.

Most dominant taxa are listed in Table 56.

Table 56: Dominant species in the zooplankton of the combined study

	species	% dominance (CYP)
1	Cyclopidae ssp. (Copepoda)	40.8
2	*Chydorus sphaericus* (Cladocera)	21.2
3	*Simocephalus vetulus* (Cladocera)	13.5
4	Nauplia ssp. (Copepoda)	3.7
5	*Chaoborus crystallinus* (Insecta)	2.9
6	*Alona guttata* (Cladocera)	2.7
7	*Eudiaptomus gracilis* (Copepoda)	2.5
8	*Arcoperus harpae* (Cladocera)	2.5
9	*Graptoleberis testudinaria* (Cladocera)	2.3
10	*Lepadella patella* (Rotifera)	1.1

Compared to the single substance approaches, more Cladocera were found in higher abundance. The Rotifers are less important in the presented community. In the single substance approaches, *Chydorus sphaericus* was present too a much lesser extent. The quite high dominance of this small-sized Cladoceran is a major difference between the zooplankton communities. This fact had a certain influence on some of the summarized parameters with respect to their no effect levels. This is discussed below in greater detail.

5.6.2 Abundance data

5.6.2a Total abundance

The abundance of all individuals that were found in the treatment levels at the sampling dates is presented in Figure 98. Between day 3 and 41 a.t. there is a treatment related loss in the abundances in levels higher than number 2. On day 7 and 14 there are more animals in level 4 than in level 3. Levels 1 and 2 show only minor increases during the whole year on some occasions. Starting on day 41, levels 3, 4, and 5 are higher than the control range. Level 3 is inside the control range again on the last two dates in the first year, the two others do not recover.

In March of the second year levels 3, 4, and 5 are increased again. Later on, only level 5 has higher numbers of zooplankton organisms. Level 4 is higher than the controls in June, 2002. Level 1 is lower than the controls in May and June 2002.

NOEC for the total abundance of the zooplankton is level 2 (0.075/16 µg/L CYP/IPU). The NECs are 0.171-0.186-0.236 µg/L CYP in the combination (n=7). This is higher than the

amount in level 2 but still lower than in level 3. Mean NEC for days 3 and 7 is 0.042 µg/L, that means even lower than level 2. All in all NEC and NOEC corroborate each other quite fine.

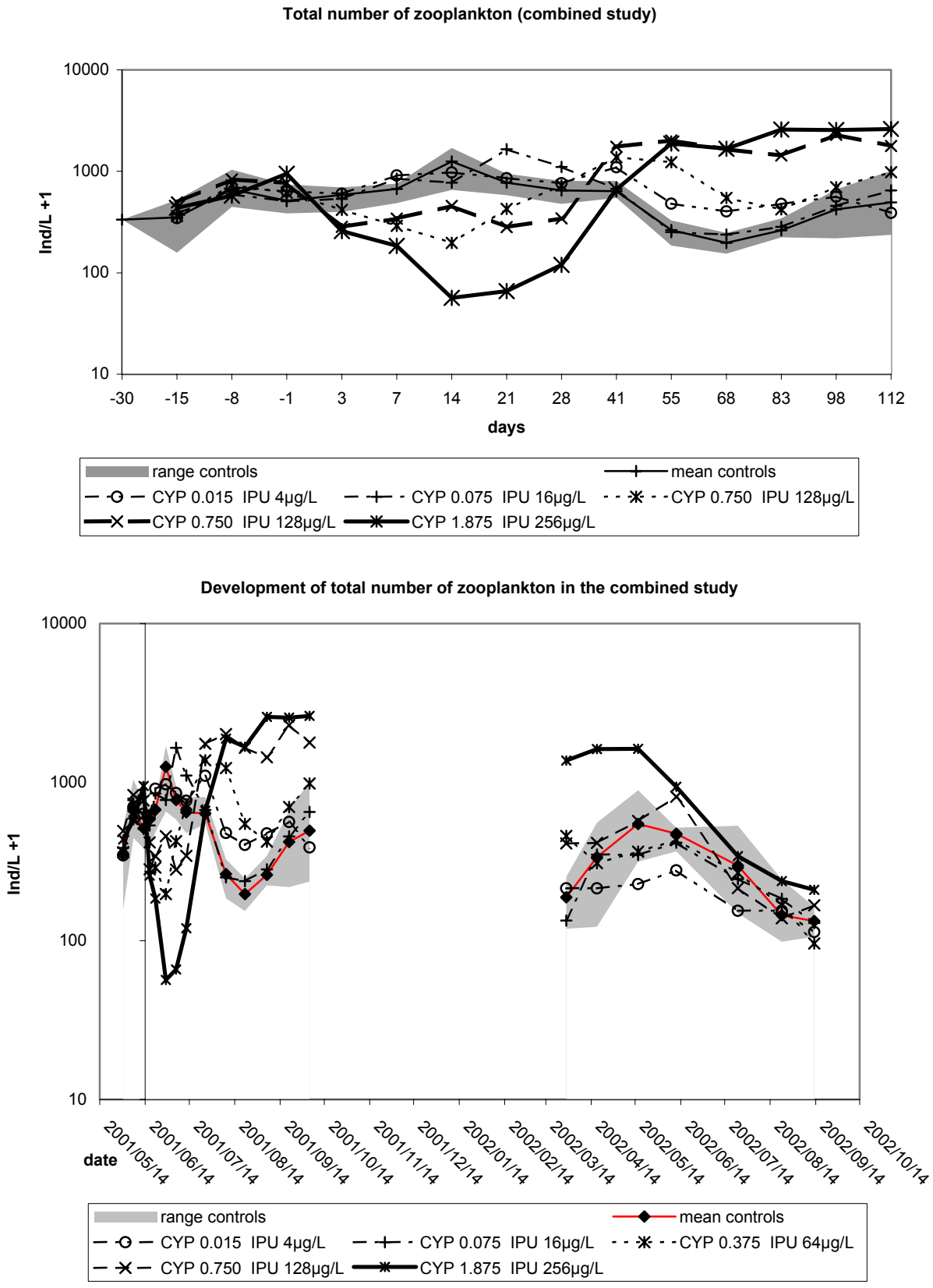


Figure 98: Total abundance of the zooplankton in the combined study; top: year one, bottom: both years

The curves can be interpreted in this way: CYP action decreases sensitive organisms shortly after the application. As already seen in the CYP study, the most susceptible species is *Chaoborus crystallinus*, the main zooplankton predator in the system. Its abundance is lowered in the same way in the combination, too (3.7.2c and 5.6.2c). Effects on *Chaoborus* are seen in all treatment levels and last until June 2002. As a consequence, predation pressure is lowered and more zooplankton is able to live in the ponds. Abundance of the predator is low enough to enable this development for level 3 and higher. As *Chaoborus* recovers, its prey is depleted. Only in level 5 this secondary effect is strong enough to last as long as it takes for the predator to be in the control range again. Decreases in level 1 in 2002 cannot be addressed to *Chaoborus* predation. This is rather to competitive action of some kind and may not be related to the treatment, because NOECs for zooplankton total abundance and for the phytoplankton community are indeed level 1 or higher.

Effects of IPU on the algae may enhance the depletion in the first month. IPU action alone caused a slight decline in zooplankton in IPU2 and higher. Here in the combination, losses in algae were found in this time slot (cf. especially Figure 92 and Figure 93) that could lead to a more pronounced CYP action. A zooplankter which is not well fed may not be able to bear as much insecticide as a well fed one.

In the two highest IPU levels zooplankton abundance increased in mid-summer of the second year, related to an increase in algae. Such an increase in zooplankton food was not to be seen in the combination and consequently no increase of the zooplankton occurs.

In the combination the NOEC is higher compared with the one in the CYP or the IPU study. The main reason is probably the different community structure of the zooplankton in the combined study. Here, the small Cladoceran *Chydorus sphaericus* plays a major role (Table 56), whereas it did not in the IPU or the CYP study. The NOEC in the single substance studies was determined mainly by *Simocephalus vetulus* (NOEC 0.075 µg/L CYP, 4 µg/L IPU), the Nauplius larvae (NOEC 0.015 µg/L CYP, <4 µg/L IPU), and *Chaoborus crystallinus* (NOEC<0.015 µg/L CYP, not sensitive towards IPU). These taxa were the most abundant ones that reacted to at least one of the pesticides at low concentrations. Thus their response lead to the impact seen in the total abundance in the single application approaches. In the combined treatment, additionally, the effects on *Chydorus sphaericus* play a major role for the total abundance due to its dominance. It is less sensitive (NOEC level 2, 0.075g/L CYP, 16 µg/L IPU) than those taxa determining the total abundance NOEC in the single species approaches. A further discussion about the development of *Chydorus sphaericus* may be looked up in chapter 5.6.2h.

5.6.2b Species richness

The number of zooplankton taxa in the combined treatment is plotted against the sampling dates in Figure 99. With the exception of day 68 a.t. levels 4 and 5 are below the control range from day 3 a.t. up to day 98 a.t.. Level 3 has less taxa on day 3 to 21 a.t. and on day 98 a.t.. Level 2 is slightly decreased on day 55 and 98 a.t..

Table 57: NEC values of the species richness in the zooplankton of the combined study

NEC	CYP [µg/L]	N	IPU [µg/L]	N
lower	0.136	5	26.31	5
middle	0.220	5	40.67	5
upper	0.420	5	71.94	5

In the CYP study, this endpoint has not been affected at all. With IPU, secondary effects started in IPU3, but they were not very pronounced. In the combination, a distinct dose-response pattern is exhibited. NECs are given in Table 57. Together with the curves they indicate a no effect level of level 2. Recovery takes up to day 112 (same time as with IPU alone).

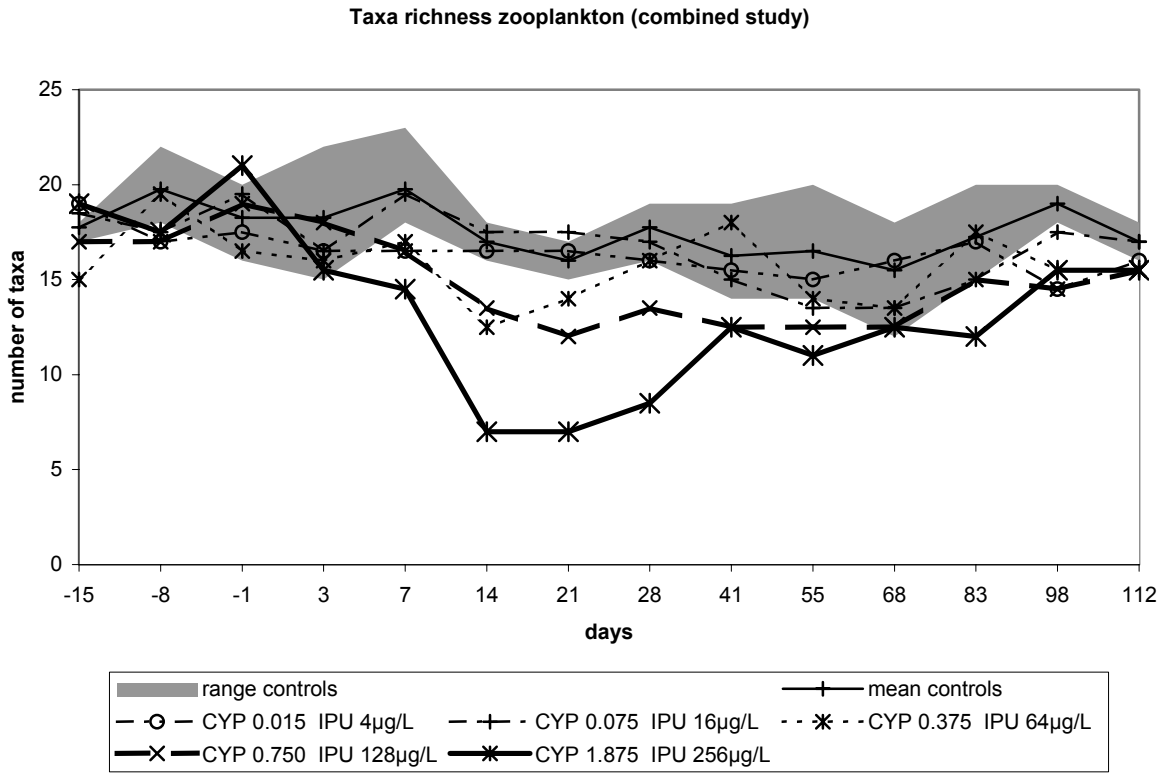


Figure 99: Taxa richness in the zooplankton of the combined study

In short, the taxa richness is more sensitive to the combined treatment than with each of the pesticides alone. The simultaneously decreased food supply (5.5.4, IPU action) on the first few days a.t. leads to the exclusion of more taxa that are not as good competitors as others under the influence of the insecticide (Table 58). Another interpretation is that these taxa, especially some Cladocera, were not able to bear as much insecticide because of poorer nutrition.

Table 58: Taxa missing in level 5 between day 14 and 21 a.t

systematic group	taxon
Copepoda	*Eudiaptomus gracilis*
Cladocera	*Chydorus sphaericus*
	Alona affinis
	Alonella nana
	Arcoperus harpae
	Graptoleberis testudinaria
Insecta	*Chaoborus crystallinus*
Rotifera	*Lecane* forma “monostyla”
	Lecane forma “diplostyla”
	Mytilina mucronata

Interestingly, Rotifers were not affected as much as with IPU alone. They are generally less sensitive to CYP (cf. CYP part) than the Cladocera. By the reduction of the Crustaceae (mainly due to the insecticide) the Rotifers seem to be better able to survive the treatment and its effects.

5.6.2c Chaoborus crystallinus

This invertebrate predator exerts a top-down control on the biocoensis of the plankton in the ponds (cf. the CYP part). It is very sensitive to the insecticide. Impact in the combined treatment are presented in Figure 100. All levels are negatively affected at least to some extent. Recovery takes place from May to June 2002. Effects are similar to the CYP study. NOECs are identical, <0.015 µg/L CYP in the combination. NECs are 0.003-0.010-0.044 µg/L CYP in the combination, which are almost exactly the values of the single CYP treatment.

Chaoborus crystallinus is so very much susceptible towards the insecticide that no secondary effects play a role for its development but the treatment. Additionally, IPU treatment had no effect on it at all. Consequently, the direct reactions triggered by the reduced predation pressure on the zooplankton grazers can be expected to be the same as with CYP alone.

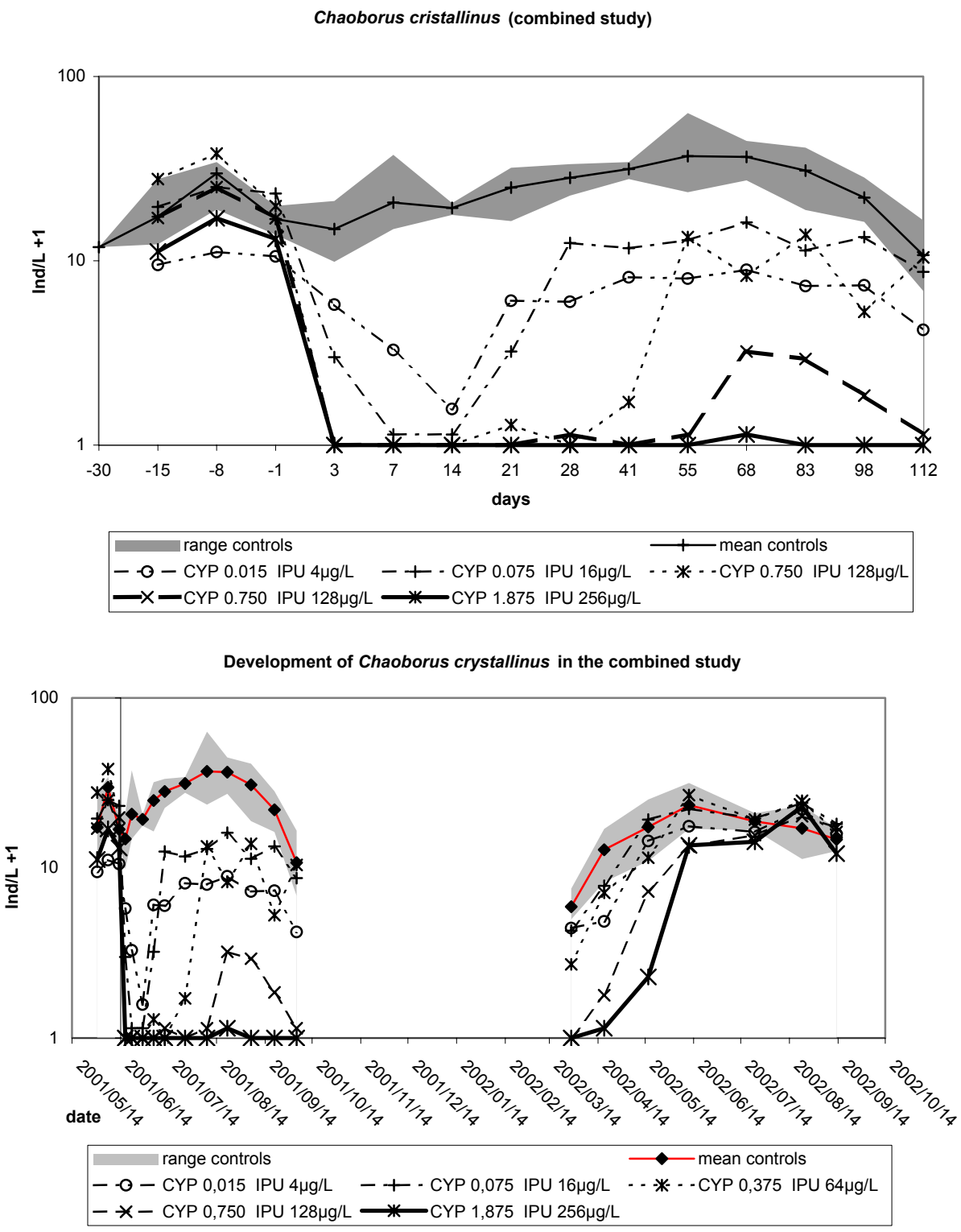


Figure 100: Development of *Chaoborus crystallinus* in the combined study; top: year one, bottom: both years

5.6.2d Nauplii

The larvae of the Copepoda were affected to a certain degree in the single substance approaches. Reaction had been not too pronounced, but they started at very low concentrations

(CYP1 and IPU1 (or less) as NOEC). The impacts were mainly secondary ones with the herbicide (decrease). Additionally, CYP exerted direct toxic effects. An impact of the combination can therefore be expected.

The development of the Nauplii is presented in Figure 101. Treatment related decreases are seen in level 3 to 5 on day 7 a.t.. On day 14 a.t. only level 5 is still negatively affected. Beginning with day 21 a.t., numbers are increased until day 83 a.t.. Highest counts are in level 3, level 2 and 4 are comparable in numbers up to day 55 when level 2 is nearer the control range. Level 5 shows the smallest increases except for days 55 and 68 a.t.. On these dates level 1 is also slightly increased.

In the second year, level 5 has a lower abundance from May to July and a higher one in August (together with level 4). All other enclosures show only minor variations for isolated sampling dates.

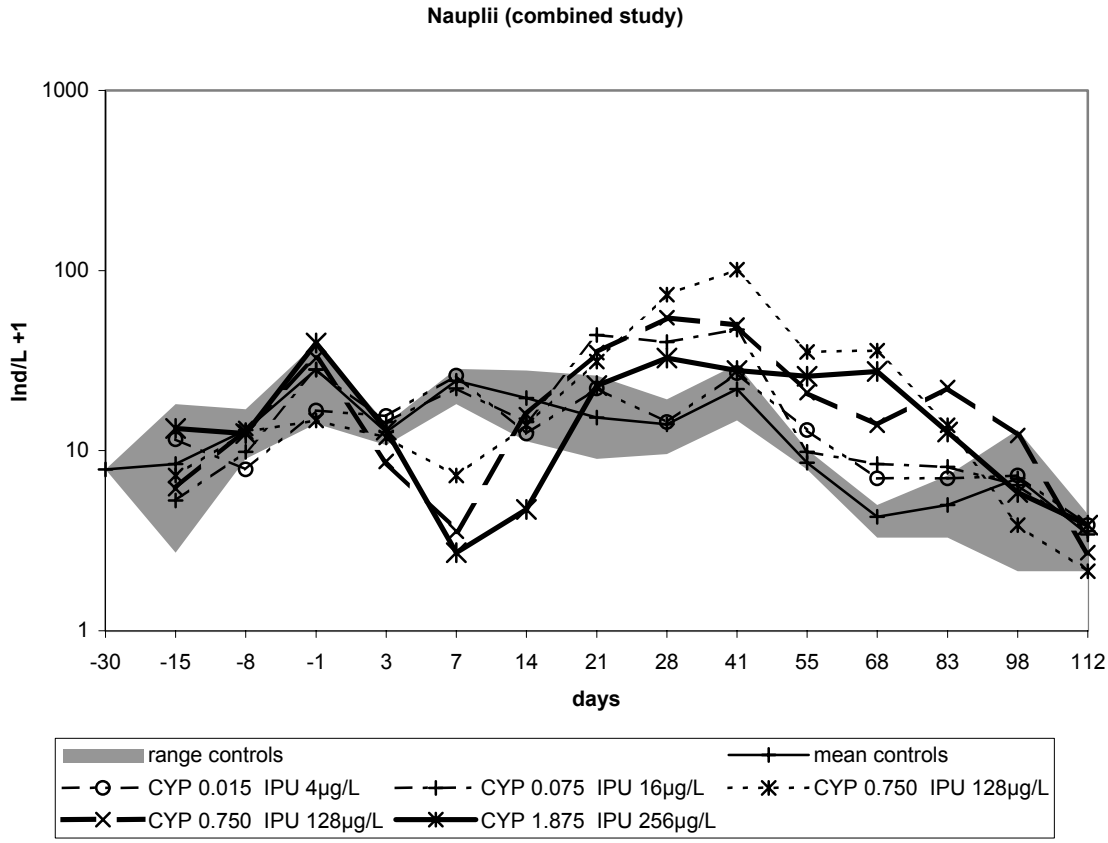


Figure 101: Development of Nauplius larvae in the combined study

NOEC is level 1, which is not backed by the NECs (1.461-1.553-1.645 µg/L CYP in the combination, n=5). This finding can be explained by the nature of the reaction to the treatment in the first year. It is certainly a secondary one, because level 3 is affected most. As a results, the linear regression design of the NEC calculation does not apply. Regression must be restricted to some of the levels that show an almost linear effect (i.e. level 1-3 or level 3-5). In any case, the values must be interpreted with great care.

The direct effects on day 7 and 14 a.t. do not define the overall NECs so much. They are clearly related to the treatment and start in level 3. NOEC is level 2 on day 7 a.t., corroborated

by the NEC (its mean is approximately 0.060 µg/L CYP in the combination for day 7 a.t.). This direct toxicity of the combined application is more pronounced than with any of the pesticides alone. Direct effects are short-termed, merely about one week (class 2 of BROCK *et al.* 2000 in EU 2002 for them alone). In any case, secondary effects can be seen up to day 83 a.t. resulting in a class 5 effect (BROCK *et al.* 2000 in EU 2002) in all enclosures (and in level 5 still in the next year).

When interpreting the data of the larvae after day 21 a.t., please also consider the reaction of the adults (cf. mainly Figure 102, abundance of *Eu. gracilis* is lower to one degree of magnitude, Figure 104). In the combined study, the adults increased rather nicely treatment related after day 28 a.t. (Figure 102). More adults normally mean more offspring. Consequently, the fact that the maximum of Nauplii is found in level 3 and not in level 5 has to be explained: Both single substance approaches had lowered abundances in Nauplii (3.7.2d and 4.7.2d). Thus, the development with the single substances is just the other way around as in the combination (after day 28 a.t.). So the relation of the abundance of Nauplii to the treatment level is the result of two opposed processes: Increasing offspring due to more adults and reduced numbers of larvae due to the pesticide action. Combining these two factors results in an optimum curve like the one found here is the consequence.

For the second year, a clear reason for the decrease in level 5 cannot be given. Here in the combination, the result of competition in a thoroughly changed ecosystem (cf. 5.3, 5.4, 5.5, and other zooplankton data) seem to be unfavorable for the Nauplius larvae. All other changes from the control range are negligible.

In short, the combined treatment caused different and more pronounced reactions in the number of Nauplius larvae. The no effect level is identical to the single substance approaches, though.

5.6.2e Cyclopoida

The development of the cyclopoid Copepoda is depicted in Figure 102. NOECs in the single substance approaches were CYP4 and IPU4.

In the combination, level 5 is decreased in Cyclopoids from day 14 to 28 a.t. Such a decrease has not been seen in the single substance studies. A reason could be a higher susceptibility to the insecticide due to less food supply (algae or zooplankton) in the relevant time slot. This combination effect is of minor importance, because it only occurs in the highest treatment level and is over-compensated by a later increase. The increases that occur in the treatment level 2 and 4 up to day 28 a.t. are too unspecific to be interpreted and merely chance effects. The development in level 1 is dealt with below.

Increases over the control range can be seen from day 41 to day 83 in all treatment levels (excluding level 2 on days 41 and 55 a.t.). Abundance in level 5 is not the highest because the toxic effects have to be compensated (days 41 and 55 a.t.). NOEC for this process is smaller than level 1, but this has to be discussed in greater detail (see below). The increases in abundances may be due to a combined treatment effect: Less predation (5.6.2c) plus better outcome of the competition under IPU influence[31]. Effects recover on day 112 a.t., fluctuations

[31] With IPU alone only level 5 showed an increase that started later.

in 2002 are discussed below. For the larvae (5.6.2d), more adults mean more offspring, of course.

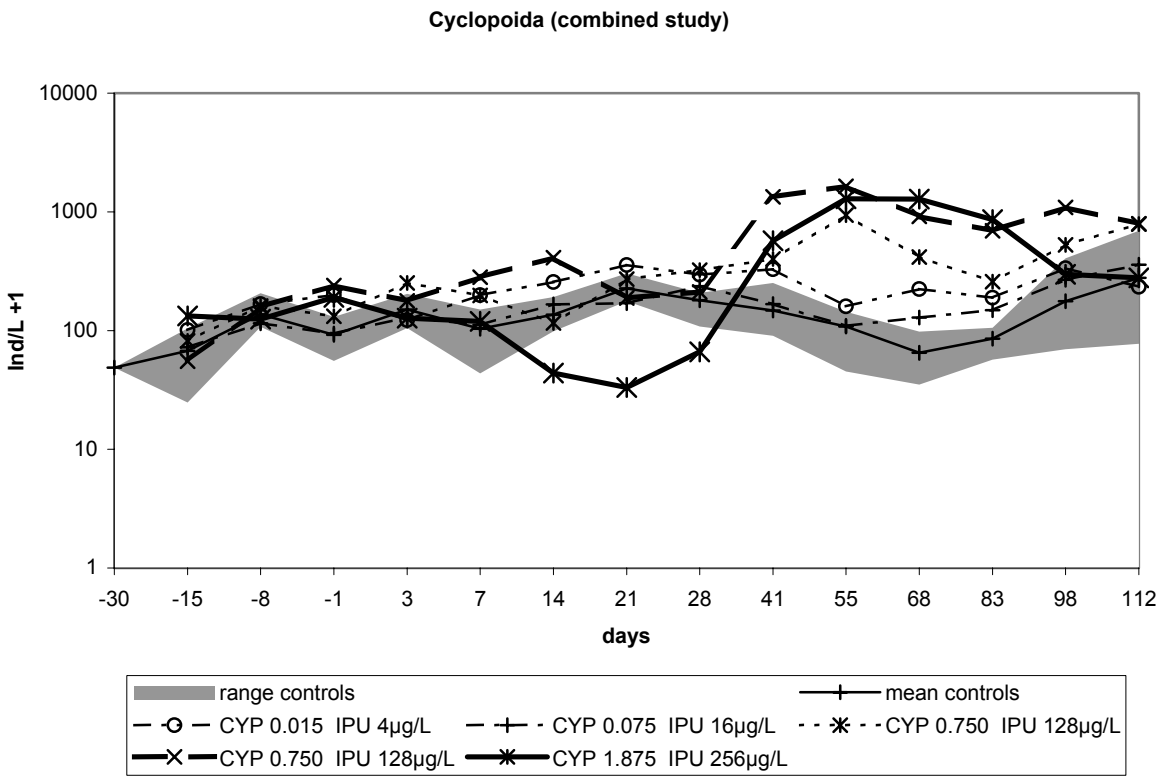


Figure 102: Development of the Cyclopoida in the combined study

As noted above, level 1 is higher than the controls most of the time (thus leading to the low NOEC). This heightened abundance seems to be a special feature of this treatment level which is already starting on day 7 a.t.. Predation pressure in level 1 is lowered by the CYP related decrease in *Chaoborus crystallinus* (5.6.2c). However, this lowered abundances in the predator was also seen in the CYP study and did not lead to more Cyclopoids there. IPU treatment alone is not hinting at an increasing effect. In the combination, algae are affected by the treatment at level 2 and higher, but not in level 1. As for the carnivorous Cyclopoids, no change in prey is seen after the treatment (5.6.2a, NOEC level 2). Consequently, a definite reason for the increase cannot be given. In any case, both enclosures with treatment level 1 showed this increase, so addressing the effect to a kind of "special quality" of a single enclosure is not possible (because both enclosure having the same "special quality" is very improbable). More probably, the combined treatment lead to a modified so-called "U-shaped" or "J-shaped" dose-response pattern (hormesis, DAVIS and SVENDSGAARD 1990, CALABRESE and BALDWIN 2002, see Figure 103). The modification is that the lowest treatment level did not show an effect that is opposed to the higher levels but even better resembles them.

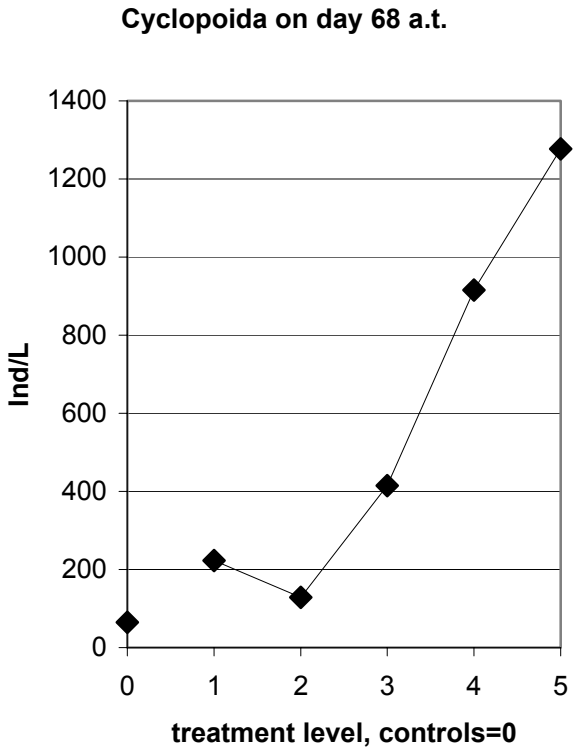


Figure 103: Modified "J-shaped" dose response pattern of the Cyclopoids (means) on day 68 a.t.

Interpretation of this behavior can be given in two ways that corroborate each other in a way:

First, the increase in level 1 is already beginning on day 7 a.t. (Figure 102). In the first month a.t. less algae are present in level 2 than in level 1 (Figure 90)[32]. During these days, the increase in Cyclopoids in level 1 compared to level 2 can build up. It is preserved for the rest of the year, because all other effects on the abundance of the Cyclopoids apparently cannot compensate for this increase.

Second, there really is a hormetic effect of the combined treatment in level 1 (starting on day 7 a.t. and lasting up to day 83). As CALABRESE and BALDWIN 2002 point out, such a "improving function" of toxicants is often seen: "In fact, so routinely was the hormetic response observed that the investigators proposed the creation of the term SC20 […] to describe the stimulatory response in low concentrations". Examples were also seen in toxicant mixtures and in ecotoxicological studies (e.g. JOY 1990 in CALABRESE and BALDWIN 2002, WALSH *et al.* 1982). A temporal component is also discussed: Hormesis can either occur "[…] via a direct stimulatory response or an overcompensation response […] via an initial disruption in homeostasis" (CALABRESE and BALDWIN 2002). In the presented case, homeostasis can be interpreted as the balance in the ecosystem that is resulting in the abundance seen in the controls. The latter reason for the occurrence of hormesis may apply in the presented study. The "disruption" would be the treatment that affects at least some endpoints at level 1 (Table

[32] Such a decrease is almost certainly due to the herbicide treatment. Secondary effects in level 2 are too weak to compensate for the losses. In the total zooplankton abundance (excluding the Cyclopoids themselves) no such development is visible. Level 1 and 2 have almost the same mean abundance.

55 and Table 62). Overcompensation must be due to reduced mortality (because the generation time is too long for a reaction as quick as seen here: 1-2 generations per year, SOMMER 1994)

Following this interpretation implies that level 2 has no effects at all (no hormesis and no toxic effects) and the mere toxicological response to the combination would result in decreasing abundance in level 3 to 5. It cannot be seen because the reduced predation leads to an increase that totally outweighs any toxic effect. Additionally, the factor time should not be forgotten: Both hormesis and the secondary increase take some time to come into being so that they get visible when the initial trigger (pesticide levels) is already lost.

In any case, such a pattern may also be seen in the conductivity (5.3.2), the pH (5.3.5), and the alkalinity (5.3.1) or the PRC on water quality parameters (5.3.7) at least to some extent. For some water quality parameters, a biological rationale for the type of reaction was worked out. Even where (or if) this rationale does not apply, the differences in the water quality between level 1 and 2 may reinforce the processes described above for the Cyclopoids.

On any account, this reaction type or intensity has not been seen the abundance of the Cyclopoids in the single substance studies. Combination effects must be noted. Yet giving a NOEC is difficult. Williams' test (that is assuming linearity in the reaction) results in a value even smaller than level 1. Keeping the possibility of hormesis in mind and remembering that level 2 is within the control range most of the time, a NOAEL (No Observed Adverse Effect Level) of level 2 is applicable. The NEC, that is assuming a log-linear relationship, is quite insensitive due to the type of reaction: 0.523-0.895-1.651 µg/L CYP in the combination.

In the second year the treated enclosures are again increased in Cyclopoids in March. Afterwards abundances decline and are lower than the controls in May and June (except levels 2 and 3). Then no more effects are visible. These effects indicate that the test systems are still (non-specifically) influenced to some extend. This is due to the long generation time of these animals (1-2 generations per year, SOMMER 1994). Consequently, effects take some time to be fully silenced.

5.6.2f Eudiaptomus gracilis

The calanoid Copepod *Eudiaptomus gracilis* reacted strongly to the combined treatment (Figure 104). Since it was not influenced by the IPU treatment, it is not surprising that its development resembles the one in the CYP study. The abundance is getting higher in the lowest treated enclosures first (level 1 on day 7 already) and the others follow some time later. By the time level 4 and 5 reach their maximum the lower treated levels are already near the control range again (day 83 and 98 a.t.). The explanation follows the one with CYP alone: Less predation in all levels (cf. 5.6.2c) lets the Copepod reach higher numbers as the insecticide declines. Interestingly, the distribution of the maximums is different from CYP treatment alone. These effects will be discussed in the next part of the presented study.

In the second year, only the levels with still lowered grazer numbers (level 4 and 5) have considerably higher numbers of *Eu. gracilis*. Recovery in mid-summer is consistent with this interpretation, too. The increases in level 1 and 2 are negligible or mere chance effects. Less than 10 individuals per liter were found and thus cannot be analyzed properly (cf. MAISE 2002).

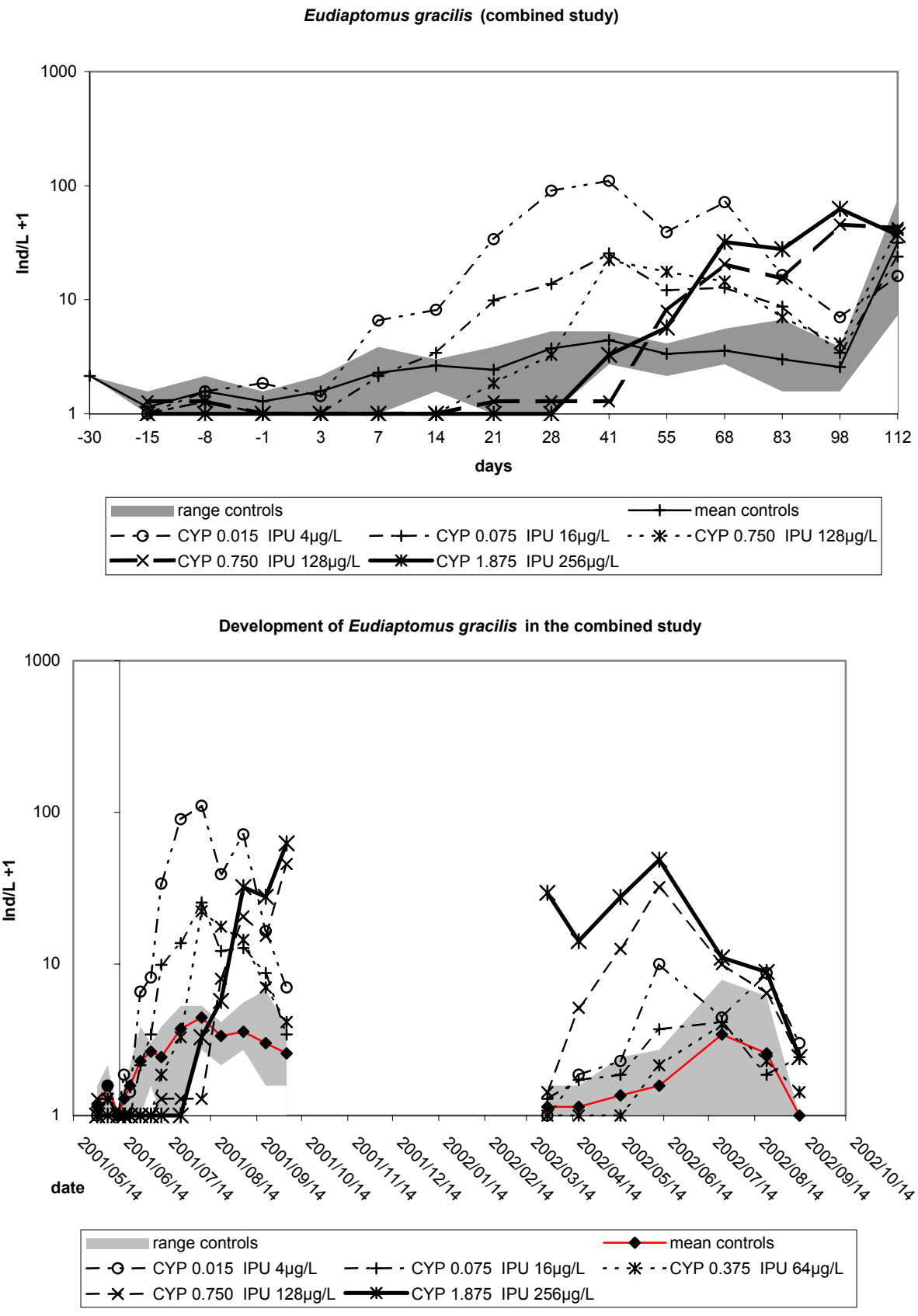


Figure 104: Development of *Eudiaptomus gracilis* in the combined study; top: first year, bottom: both years

With this clear secondary effect causing the development in the abundance, NOEC calculation is not very sensitive: level 3 with 0.375 µg/L CYP in it. NEC corroborates this value, 0.480-0.553-0.658 µg/L CYP in the combination (n=8). In the single substance study, the NOEC was level 1 (0.015 µg/L CYP). When looking at the curves in the combination again (Figure 104), effects in level 1 are quite obvious. Statistics seem to be too insensitive for this type of reaction to the treatment. A no effect level of smaller than level 1 may be assumed.

5.6.2g Simocephalus vetulus

In the single substance approaches this big Cladoceran was one of the main grazers in the system. It is sensitive towards CYP (NOEC 0.075 µg/L with CYP alone) and was influenced secondarily in the IPU study (NOEC 4 µg/L IPU).

In the combined study, it reacted mainly in the way as with CYP alone (Figure 105). Treatment related decreases in the abundances start in level 3. Recovery is seen on day 41 except for level 5. This treatment level is already higher than the controls on the next sampling date, though (day 55 a.t.). Abundance in level 4 and 5 is increased on day 55 a.t. and 68, additionally in level 5 on day 83 a.t..

In the second year, only minor variations are visible.

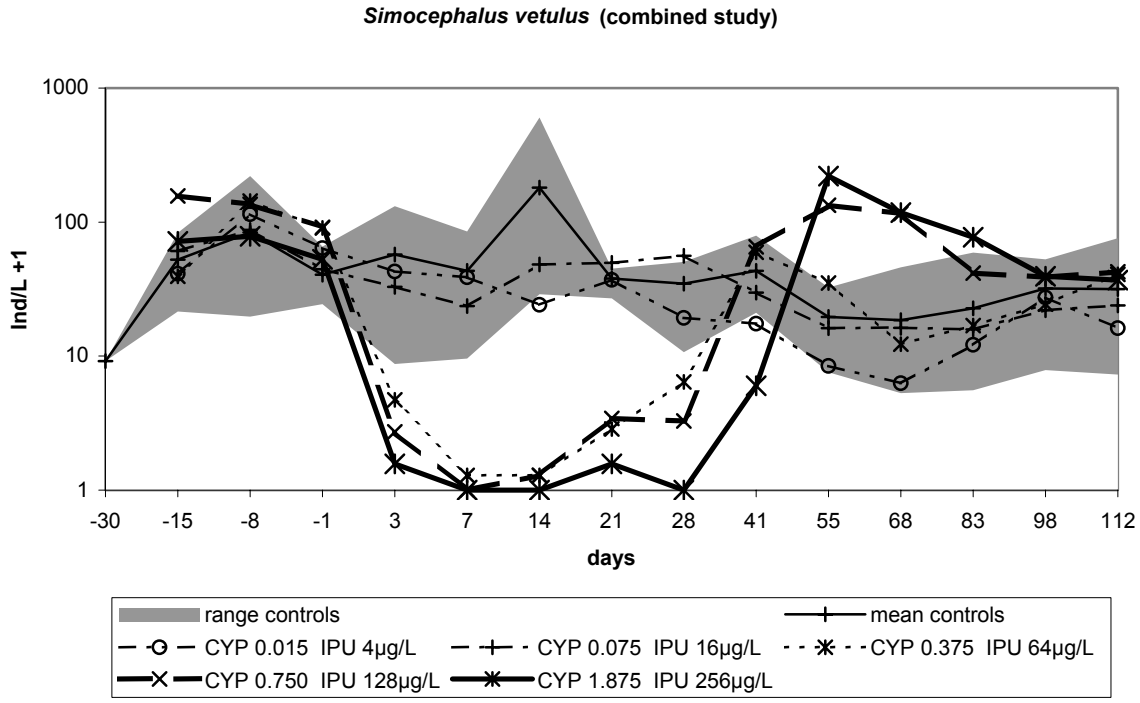


Figure 105: *Simocephalus vetulus* in the combined study

NOEC for *Simocephalus vetulus* is level 2 containing 0.075 µg/L CYP (NEC 0.149-0.268 µg/L CYP in the combination, n=8). This value is identical to the single substance study. Even the recovery time (about one month) matches. Impact is hence mainly exerted by the insecticide. IPU effects on the algae (5.5.2) were limited to the time the Cladoceran is affected by the insecticide and are balanced in the following time. Through this development, no strong secondary effects due to effects on the phytoplankton can be seen. The increases after day 41

a.t. are more pronounced than with CYP alone (predator induced, see 5.6.2c); even level 4 shows this increase here. An interpretation is that such an additional oscillation is due to a generally greater disturbance of the ecosystem due to the reduction of the macrophytes (5.4). *S. vetulus* can take a bigger advantage of the reduced predation under these circumstances than without them. Possibly, more algal biomass, which is grazed instantaneously by the Cladocera (see also 5.6.2h), can be produced, so an increase cannot be seen in the algae themselves but merely in the grazers.

In short, *S. vetulus* is not affected very differently by the combination of the insecticide and the herbicide than with the insecticide alone.

5.6.2h Chydorus sphaericus

This small Cladoceran was present in a much higher abundance in the controls than in the single substance approaches in the first year. Moreover, in the CYP and the IPU study abundances were so low that no thorough evaluation of the data was possible in that period of time. Consequently, special combination effects in the first year cannot be derived from the data.

In Figure 106 the development in the combined study is presented. A clear treatment related decrease is seen in the first month in levels 3 to 5 (up to day 41. a.t.). After day 55 a.t. the controls drop to a lower level (in July). This is a seasonal effect that is also seen in the second year in the controls of the combined and the single substance studies.

Due to this development, all treated levels are higher than the controls up to day 112 a.t.. Real increases, however, can only be seen in level 4 and 5. The others simply do not decrease so much. The inverted intensity in the effect between level 1 and 2 may be a chance effect, because the abundance in level 2 is quite low. In the beginning, abundances were almost identical for a long time. In the second year, level 4 is increased up to June and level 5 even up to August.

Effects up to day 41 a.t. are clearly related to the insecticide. As it declines, recovery can take place. The increases or lower decreases afterwards are a secondary effect of reduced predation of *Ch. crystallinus* (cf. 5.6.2c). *Chydorus sphaericus* is one of the main prey organisms of the invertebrate predator (DODSON 1974 in VANNI 1986, SWIFT 1992). Therefore, even the secondary effect is mainly related to the insecticide treatment. Additionally, the speculations about algal biomass production and its delivery to the grazers (see above) also apply here.

A secondary effect of the IPU treatment was a reduction in abundance in the second year (4.7.2h). This cannot be seen here. Merely the increases are preserved until the predator is in line with the controls again. This development is to some extend comparable to the CYP study results in the second year (a little more pronounced).

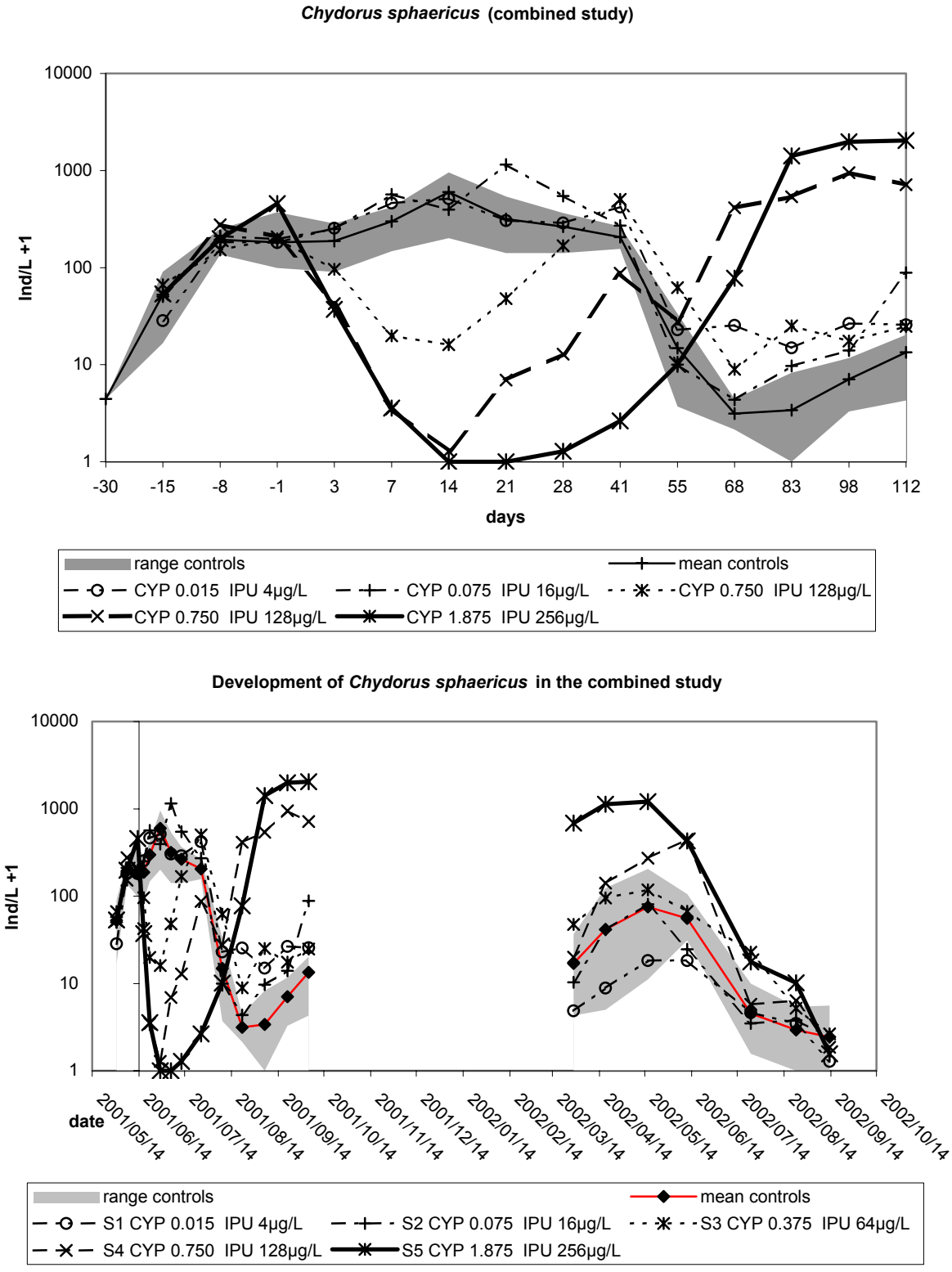


Figure 106: Development of *Chydorus sphaericus* in the combined study

NOECs cannot be compared because they are to be derived from different effects. In the combination it is level 2 (0.075 µg/L CPY in the combination). This value was derived from Williams' tests in the first month a.t.. The increases had a higher value (level 3).

NECs are higher (about level 3) than the NOEC, 0.332-0.430-0.603 µg/L CYP in the combination (n=10). The lower NOEC is taken into account, because level 3 is clearly influenced.

For the increase, the NOEC is level 3. With CYP alone, it was CYP4 (0.750 µg/L). So the combination may have lead to a more distinct reaction. Yet the data of the CYP study must be regarded as not too sure.

To sum it up, here again the Cladoceran is mainly influenced by the insecticide. The combined treatment did not affect the phytoplankton as much as the IPU treatment alone, so the CYP effects become visible very clearly.

5.6.2i Rotifera

The Rotifera in the combined study did not react in a very distinct way. Deviations are presented in Table 59. The decreases in level 5 may be due to a toxic action, those in levels 1 and 2 are rather due to competition effects. All in all, impact is too small to be interpreted in further detail. This is in line with the single substance approaches, where also only minor effects were visible. Secondary interactions in the IPU study caused greater deviations than in the CYP approach. The no effect level for IPU was around 16 µg/L, the NOEC for CYP 0.750 µg/L. For the combined approach, no NOEC could be calculated. NEC values are approximately 0.250 µg/L CYP in the combination (n=4, a little lower than level 3).

Table 59: Deviations of the abundance of Rotifers from the control range in the combined study

days a.t.	level	direction
14	4, 3, 5	decrease
21	5	decrease
28	1, 2	decrease
41	1, 2	decrease
55	4, 5	increase

In 2002 level 5 was increased in August and September; this again may be a ecosystematic effect due to the re-growth of the macrophytes (5.4).

5.6.3 Community analysis

5.6.3a Shannon index and evenness

Mean value of the Shannon index in the controls is 1.78; H_{max} is 3.09. The mean value in the treated enclosures is 1.38 (H_{max}=3.04). Medium evenness in the controls is 0.63; in the treated enclosures a mean of 0.52 was calculated.

The values of the controls are comparable to those of the single substance approaches. In the treated ponds, the mean values are lower in the combination. Figure 107 depicts the development of the evenness in greater detail.

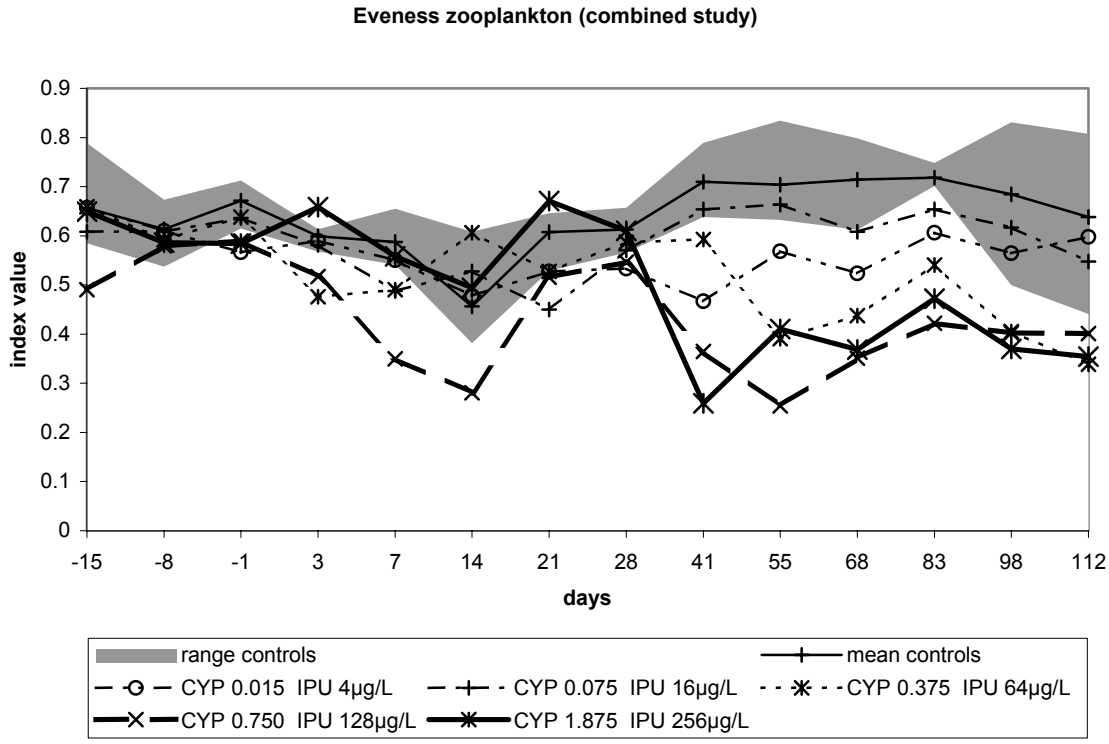


Figure 107: Evenness of the zooplankton in the combined study

Up to day 28 a.t. no distinct treatment relation is visible. Level 4 is decreased all of the time. The other levels show some smaller alterations on a few sampling dates.

From day 41 to day 112 a.t. treatment relation is better. The switch between level 1 and 2 (as in the Cyclopoids, 5.6.2e) can also be seen here. Development in the Cyclopoids, *Eu. gracilis* and *S. vetulus* (level 1 and 2 inside the control range, but level 1 lower than level 2, Figure 105) may contribute most to this development. NEC values are presented in Table 60.

Table 60: NEC values of the evenness of the zooplankton of the combined study

	NEC for CYP [µg/L]	N	NEC for IPU [µg/L]	N
lower	0.419	8	36.3	7
middle	0.573	8	37.9	7
upper	1.262	8	38.5	7

As already noted with the Cyclopoids, these values may be much too high because of the type of reaction. A NOAEL of level 2 seems appropriate. This is similar to the IPU study (16 µg/L herbicide) but higher than with CYP alone (smaller than 0.015 µg/L).

Comparing the reaction of this endpoint to the single substance studies, the impact is much more pronounced, though. As noted with the IPU study data, the endpoints evenness and Shannon index need rather big changes in the community to show a reaction. Thus, the community structure in the combined application study is changed more thoroughly than in any of the single substance approaches. This may partly be due to the higher importance of *Chydorus sphaericus*, but combination effects were seen in some other endpoints as well (e.g. Cyclopoids, taxa richness)

5.6.3b RAD index

In the single substance approaches this distance index was very apt to detect deviations due to the treatment. In the combined study, this sensitivity was proven again (Figure 108).

For level 3 to 5 a clear treatment relation can be seen up to day 404 a.t.. There is no recovery in level 5. For level 1 and 2, the inversed relation (to the "normal" expectation) is seen for 11 sampling dates over both years. A smaller application of the pesticide combination (level 1) altered the zooplankton community more severely than a more extensive one (level 2). Even level 3 reacted less pronounced (i.e. it is closer to the control range, days 41, 55, 83, 362, 404, 434 a.t.). Such a development could be expected from the results of the analyses presented above.

An explanation for the RAD pattern cannot be given, because with the RAD a mere "distance" measure between the treatment levels and the controls is calculated. Whether a general increase or decrease in some parameters is the reason for this dissimilarity cannot be derived from the curves.

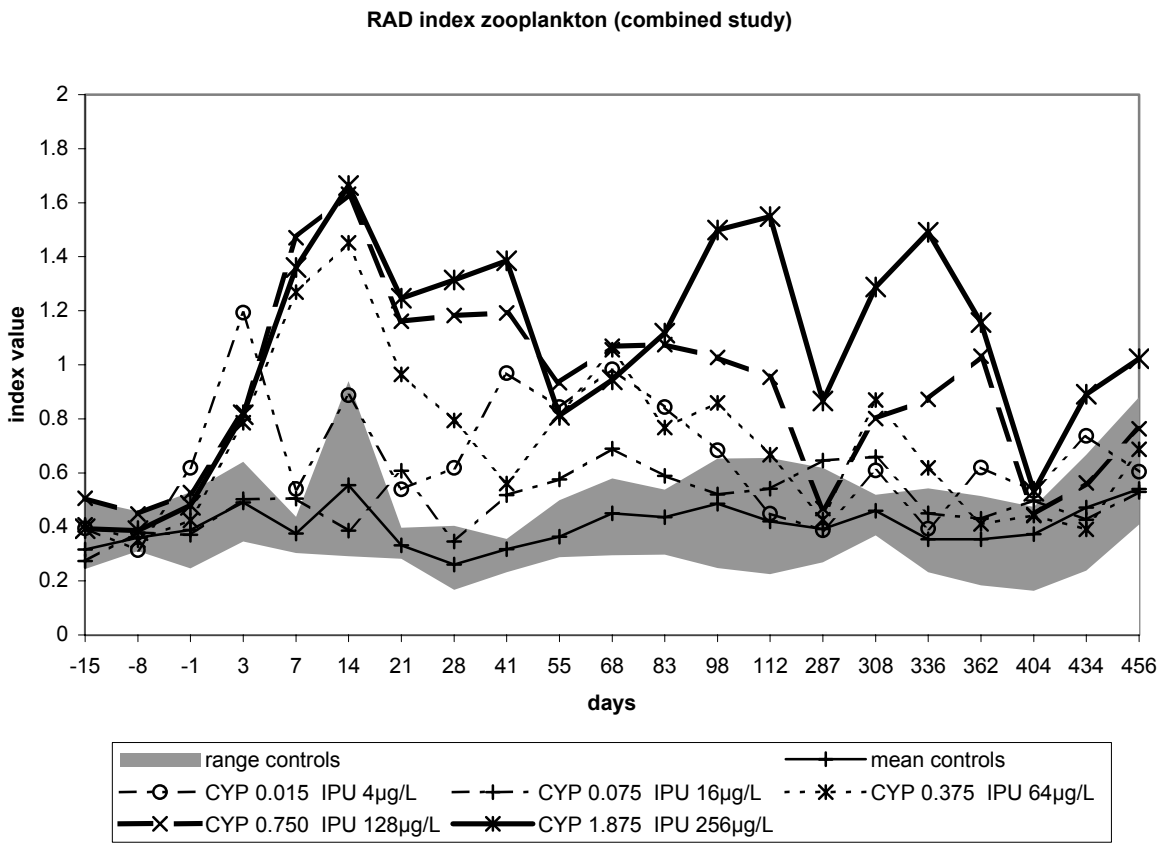


Figure 108: RAD index of the zooplankton in the combined study

NEC values match quite well with those of the IPU study: 10.1-15.6-23.1 µg/L IPU in the combination (n=11). With IPU alone it was about the same range (9-21 µg/L IPU). Again the problem here is the reaction of level 1 with even lower pesticide content than the low value of the NEC. There are effects in level 1 that cannot be detected by the NEC calculation that assumes linearity. By this process, the calculated NEC would rather become a NOAEC, but

whether the effect in level 1 is adverse (or is not) cannot be derived from the RAD value (see above). For safety reasons, no effect level of less than level 1 must be used.

As a result of the RAD analysis it can be concluded that all combined treatment levels altered the zooplankton community. In the single application approaches, at least the lowest level showed no deviations. Consequently, the combination of CYP and IPU affects the zooplankton community more severely.

5.6.3c PRC analysis

The results of this multivariate approach are presented in Figure 109. Up to day 41 a.t. treatment effects are very pronounced in level 3 to 5. Afterwards, the reaction of the zooplankton community to the treatment heads towards the opposite direction. Level 3 already starts this reversal on day 41 and 55 a.t., level 4 and 5 follow on day 55 a.t. From day 68 to the end of the first year deviations are restricted to level 4 and 5.

The development seen here can be explained by a direct toxic action in the first month and secondary but still treatment related influences afterwards. Such a development was seen in some Cladocera and the Copepoda.

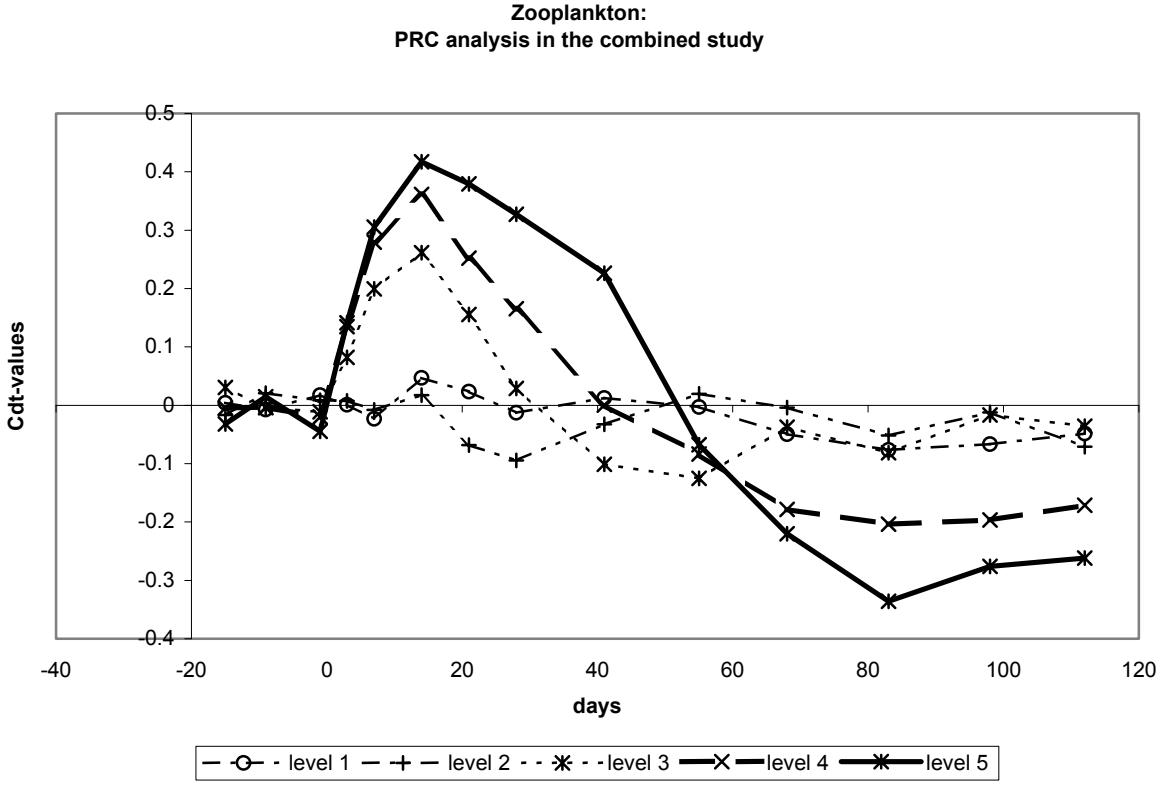


Figure 109: PRC diagram of the zooplankton in the combined study

This analysis is significant, p=0.005. It explains 46.5% of the variances by the treatment of which 40.3% are displayed. 29.3% of the variations are explained by the sampling day. Table 61 lists the relevant taxa in this analysis. Surprisingly, *Chaoborus crystallinus* is not one of those species. As shown in 5.6.2, its development to the treatment influences other taxa secondarily rather intensely. It is concluded that

1. PRC analysis is unable to detect even extraordinarily sensitive taxa when strong secondary effects as they were seen here determine the reaction of the community;
2. PRC analysis alone cannot explain secondary effects in an ecosystem.

It is thus necessary to conduct the "classical" approaches as well. Multivariate analysis does not save the trouble of looking closer at all data separately.

In any case, the PRC was able to detect some of those species that were found to have a strong impact on the community reaction (*Chydorus, Simocephalus, Eudiaptomus*) and additionally found two other Cladocera (*Arcoperus, Alona*) that have not yet been investigated in greater detail. *Arcoperus harpae* was missing right after the treatment (Table 58), *Alona guttata* has not been discussed at all. It was present in rather low abundance but was affected negatively by the treatment between day 7 and 28 a.t. (all levels but level 2). Level 5 was increased on day 55 a.t. over the control range. Consequently, PRC analysis was able to detect a susceptible taxon that would otherwise not have been discussed in greater detail.

Table 61:Relevant zooplankton taxa in the PRC of the combined study

taxon	species score
Chydorus sphaericus	-1.8910
Simocephalus vetulus	-1.1847
Arcoperus harpae	-0.7708
Alona guttata	-0.5872
Eudiaptomus gracilis	-0.5780

$NOEC_{community}$ is level 2 (0.075 µg/L CYP, 16 µg/L IPU), corroborated by the NEC (0.158-0.159 µg/L CYP in the combination, n=8). Compared to the values of the single substance approaches this value is higher than with CYP alone (smaller than 0.015 µg/L CYP) and lower than with merely IPU (64 µg/L IPU). This finding is no surprise, because

1. PRC was not able to detect *Chaoborus* in the combined approach as being sensitive→NOEC must be higher than in the CYP study
2. IPU alone had only secondary effects that cannot be investigated too well by the PRC.

However, the curves show a very much more distinct impact of the combined treatment than any of the single pesticide application analyses. It is (again) concluded that the combined approach does not alter the threshold value for an impact too much but whenever such an influence is exerted, it will be more distinct than with a single substance.

5.6.4 Overview of treatment effects of the combined application on zooplankton

Table 62 summarizes NOEC data for selected endpoints of the zooplankton of the combined study. In the following the treatment effects of the combined application are resumed.

Table 62: Summary of NOEC data of zooplankton parameters (combined study)

endpoint	NOEC [CYP/IPU µg/L]	direction of pesticide influence on data /remarks
Chaoborus crystallinus (Insecta)	< 0.015/4 µg/L	decrease, only CYP action
Chydorus sphaericus (Cladocera)	0.075/16 µg/L	decrease for one month a.t. then increase (less predation)
Cladocera	0.075/16 µg/L	dominated by *S. vetulus* and *Ch. sphaericus*, number of taxa is reduced
Cyclopoida (Copepoda)	< 0.015/4 µg/L (does not apply)	possibly hormetic effects, NOAEL of level 2
Eudiaptomus gracilis (Copepoda)	0.375/64 µg/L	increase, NOEC is too high, even level 1 deviates from the control range
Nauplii (Copepoda)	0.015/4 µg/L	secondary effects, not linear with treatment level
Rotifera	almost unaffected	
Simocephalus vetulus (Cladocera)	0.075/16 µg/L	only CYP effects, increases after one month a.t. possibly due to simultaneous effects on primary producers
total abundance	0.075/16 µg/L	determined by *S. vetulus* and *Ch. sphaericus*, same reaction type
$NOEC_{community}$	0.075/16 µg/L	two opposed reactions of the community after the treatment: up to one month a.t. and the time later

The total abundance was lowered in the first month and then increased until the end of the first year. Abundance in level 5 was still higher than the controls until the predator *Chaoborus crystallinus* had recovered completely (in June 2002). The increase was clearly related to less predation. This effect had also been visible with CYP alone (3.7.2a). The combination of the pesticides had higher NOEC than in the single substance studies. This can be explained by a different community structure, especially a higher abundance of *Chydorus sphaericus*.

The taxa richness was more severely affected than in the single substance studies. Especially less abundant Cladocera were missing at least for some time a.t.. Level 2 is the NOEL. Effects were more pronounced as in the single application approaches and started at a lower dose level.

Chaoborus crystallinus reacted exactly as in the CYP study. This insect larva is so susceptible to the insecticide that no other effects had detectable influences on its development.

The Nauplii exhibited a secondary increase instead of the decrease with IPU alone. The NOEC is identical to single substance approaches, although the larvae were reacting more intensely. Recovery could be seen after more than 8 weeks (day 98).

The Cyclopoids showed clear combination effects. In the first month, abundance in level 5 is decreased. Such a decrease has not been seen in both single substance approaches. Moreover, a distinct secondary increase after one month is visible. NOEC is smaller than level 1. This high sensitivity is cause by a modified hormetic (J-type) reaction of the abundance. The numbers in level 1 were higher than the ones in level 2 (which is within the control range). It is concluded that a NOAEL of level 2 is appropriate.

Eudiaptomus gracilis has a NOEC of level 3. Its abundances had a kind of bi-modal distribution. This is alike with CYP alone, but level 1 in the combination was clearly more influenced by the combined treatment (increases in numbers). It is concluded that the calculation of the NOEC lead to a false level because the type of reaction is not conform with the model behind the NOEC (assuming a linear trend in the reaction). The distribution of the maxima is different from the CYP study. This effect will be discussed later (see the next part of this thesis).

Simocephalus vetulus reacted similar to the combination as to CYP alone: its abundance is decreased. Additionally, a short term increase after one month a.t. in level 4 and 5 is seen. A reason for this may be the changes in the macrophytes and resulting ecosystematic effects: better growing conditions for the algae; changes in phytoplankton abundance is not visible because it is directly utilized by *S. vetulus* for an increase. The close link between *S. vetulus* and the phytoplankton has been demonstrated in the IPU study (4.8).

Chydorus sphaericus exhibited the following treatment effects: A decrease at the beginning and an increase over the control level after one month (reduced predation, ecosystematic effects of less macrophytes). Clear-cut combination effects cannot be noted, because abundances in the first year of the single substance approaches were too small. Secondary effects of the IPU treatment in the second year (decrease) cannot be seen. NOEC is level 2.

Community analysis showed that the evenness and the Shannon index were both influenced more strongly by the combined treatment than in the single substance studies. Statistically significant effects started in level 3. This is higher than with CYP alone but lower than with IPU. The differences in the communities may play a major role here. *Chydorus sphaericus* was much more abundant, so the high sensitivity of *Chaoborus crystallinus* could not play such an important role as with CYP alone. Consequently, effects on the community as indicated by the evenness begin at a higher level. With IPU alone, no direct toxicity has been seen, so only secondary actions took place in the single application study with IPU that needed a greater impact (i.e. more pesticide) to propagate through the trophic levels of the test system.

The RAD index indicated deviations in all treatment levels. The combined treatment had a more severe impact on this endpoint that the single substance approaches.

PRC analysis clearly showed the switch in importance between the direct toxic action of CYP in the combination between day 3 and day 41 a.t. and the secondary effects afterwards. Important taxa (and two additional Cladocera) were found by this analysis. Interstingly, *Chaoborus crystallinus* was not detected to be sensitive to the combined treatment. Thus, the

$NOEC_{community}$ is higher than with CYP alone (level 2, 0.075 µg/L CYP instead of 0.015µg/L). It is concluded that multivariate analysis alone cannot detect all important treatment effects, especially in a more complex scenario with heavy secondary interactions.

In short, the combined treatment exerted effects on the zooplankton that resembled those of CYP treatment alone. Alterations can be explained by (secondary) IPU action. With CYP alone, the NOEC for all zooplankton data was set to 0.015 µg/L or lower. The NOEC of the combined treatment is identical. Please note that all treatment effects were more distinct in the combined application, though. The impact on the zooplankton may therefore start at even lower concentrations as with the insecticide alone, because the impact of the combination is stronger. Further research with lower treatment levels is needed to give an answer to this question.

5.7 Summary of the combined study effects

The planned pesticide levels were met quite nicely in the combined approach (via direct analysis for IPU and biomonitoring for CYP). DT_{50} values correspond to those of the single substance approaches: about 15 days for the herbicide (detoxification of the water column after about nine month in level 5) and about 3 days for the insecticide (derived from the biomonitoring data of *Chaoborus crystallinus*).

Biomonitoring with *Eudiaptomus gracilis* (Copepoda) and *Simocephalus vetulus* (Cladocera) corroborated the EC_{50} data of the CYP study at least to some extend. The Copepod is less sensitive than the Cladoceran. Differences in the LC_{50} data may well stem from test factors that could not be controlled at the Grünschwaige research station. Performing such biomonitoring experiments under more standardized conditions is recommended. However, effects of the herbicide could not be seen here. The toxic action was determined solely by CYP.

Biomonitoring with *Chaoborus crystallinus* lead to convincing results. This very susceptible animal reacted exactly in the same way as with CYP alone. IPU study results indicated no effect of any kind of the herbicide treatment at all. Results in the combined treatment biomonitoring showed that no direct CYP action is present in the system after 41 days. Taxa that are less susceptible will not be influenced any more by the insecticide even earlier.

Biomonitoring data of *Chaoborus crystallinus* was also used to calculate the insecticide residues in the water column. Using a given LC_{50} value lead to convincing results. This promising approach may be worked out in greater detail. Advantages are supposed to be greatest when pesticide levels are below the detection limit of the chemical analysis - or this analysis is very expensive or difficult. Required for this approach is a species that is very sensitive to the active ingredient and for which well-know and assured toxicological data is available.

Water quality parameters reacted to the herbicide in the combination. NOEC was 16 µg/L IPU in the combination. NOEC of the combination was higher than with IPU alone (4 µg/L). The reason for this is an insecticide action on grazers that secondarily lead to less impact on the algae (possibly helped by some differences in the macrophyte cover). Therefore, more herbicide is bearable for the system. This is a clear combination effect that even allows some

more pesticide residues in the water column without changing the physical and chemical properties of the water in the test system. This finding may not be generalized without further research because it is specific to the test system used. Effects can again be summarized as a DO-pH-alkalinity-conductivity syndrome.

Macrophytes had a NOEC of level 2 (16 µg/L IPU, 0.075 µg/L CYP), which is lower than with IPU alone (64 µg/L, IPU3). The reason may be a slightly different species composition and abundance in the test systems. Differences between level 1 and 2 may also stem from effects of a different stock of macrophytes.

The phytoplankton in the combined study showed major deviations from the control range only for about 1-2 months. More intense effects of the CYP influence on the algae (direct or indirect) were observed but they came about some time later than with the insecticide alone. IPU action lead to this delay. Later on, no more effects were visible. The taxa composition was different in level 1 compared to all other treatment levels (higher percentage of Chrysophyceae), so that at least for some parameters an explanation for the inversion in the intensity of the treatment effects between level 1 and 2 could be given. RAD index is the only endpoint that did not show complete recovery within two months. The intensity of all effects seen here was higher than with the single substances. NOEC for all phytoplankton endpoints is set to level 2 (identical to IPU alone, but higher than with CYP). In short, the phytoplankton composition is altered for a shorter period of time compared to the IPU study (no oscillations up to winter) and reacts less sensitive to the combined treatment (compared to the CYP study). However, when effects occur, they are more pronounced. These effects can be summarized as a short-term decrease due to IPU-sensitive algae at first and as an over-compensation due to reduced grazing (CYP action) later on. Other secondary effects are balanced in the test system used here. Such a fast silencing may be a special feature of the macrophyte-dominated pond system and should not be generalized for arbitrary insecticide-herbicide combination treatments.

The zooplankton NOEC is calculated to level 1 (or lower). Combination effects were seen in several endpoints; abundances were reduced in the first months by CYP action and were afterwards even higher than the controls (e.g. total abundance, *Simocephalus vetulus*, *Chydorus sphaericus*, Nauplii). Reduced predation due to CYP action on *Ch. crystallinus* is one reason for this development. It can additionally be supported by an increase in algal production that cannot be seen because it is directly grazed by the increased numbers of zooplankton grazers. Such a more intense growth in algae could be facilitated by the better nutrition[33] of the algae in the time slot about 2-3 months a.t. (due to damaged or decaying macrophytes). This can be the reason why such an (intense) increase was not seen with CYP alone.

The very CYP sensitive *Chaoborus crystallinus* exhibited exclusively CYP effects. This finding corroborates the assumption made in the concentration estimation for the insecticide residue via the biomonitoring. NOEC was level 1 (with 0.015 µg/L CYP) or lower.

[33] Changes in several abiotic parameters in were observed (5.3), but not in all. Re-cycling of nutrients can be very fast, especially for phosphates (about 10 minutes, LAMPERT and SOMMER 1993). So nutrients released by the macrophytes due to IPU action may well be directly taken up by (more resistant) algae. The sampling scheme in the presented studies may therefore well be unable to detect such redistributions.

Eudiaptomus gracilis showed a similar behavior as with CYP alone: Increases in the lowly treatment enclosures at first and later on the highly treated one as well. Alterations in this pattern between the CYP and the combined study are discussed in the next part of the thesis.

Cyclopoids and taxa richness were not or only slightly influenced by the single substance treatments, respectively. In contrast, they showed effects in the combination. Particularly some Cladocera species were missing after the treatment. The Cyclopoids were reduced right after the application in level 5 only and increased after the first month in all treatment levels. Interestingly, level 1 had a higher abundance than level 2. This may be a special combination effect (hormetic or "J-shaped" type of reaction).

Community analyses revealed that the impact of the combined treatment can be divided in two phases:

1. Direct toxicity of CYP (decreases) and its compensation in the first month and
2. secondary effects (increases) due to reduced grazing facilitated by indirect IPU action later on.

RAD index revealed that level 1 is influenced more severely than level 2 for several sampling dates. The Cyclopoids, for example, had a higher increase in abundance, and *S. vetulus* a higher decrease. An explanation was tried to be given where possible. In any case, a low-level combined treatment altered the community more intensely than a higher one.

NOEC is the same as with CYP alone, mainly because of the high susceptibility of *Chaoborus crystallinus*. The mentioned combination effects and the fact that most impacts were stronger (at the treatment level they appeared) than with any of the pesticides alone strongly suggests a greater disturbance of the test system by the combined treatment.

A graphical interpretation of the combined action on the test system is given in Figure 110. The curves may not be interpreted in a way that for example there are more grazers than phytoplankton. The relationship of the curves should be looked at in respect to their relative development. The curves are subdivided (arranged) in those parameters that are more intensely directly affected by CYP (upper part of the diagram) and those that are (more) directly influenced by IPU (lower part).

An example for an interpretation is: IPU (direct impact) lowers the phytoplankton numbers. Simulatneously CYP (direct effect) reduces the numbers of grazers and predators. As a result, phytoplankton numbers increase (while there is still CYP and IPU in the water column). By the time the direct toxic CYP impact ends (dotted line), the abundance of the grazers increases (secondary effect!). Predation pressure is still lowered. Consequently, the abundance of the phytoplankton is limited by a top-down process (grazing; while there are still IPU residues in the water). Hence, in the period of time on the right of the dotted line, secondary interaction in the food web have a greater impact on the development of the ecosystem than the pesticides have.

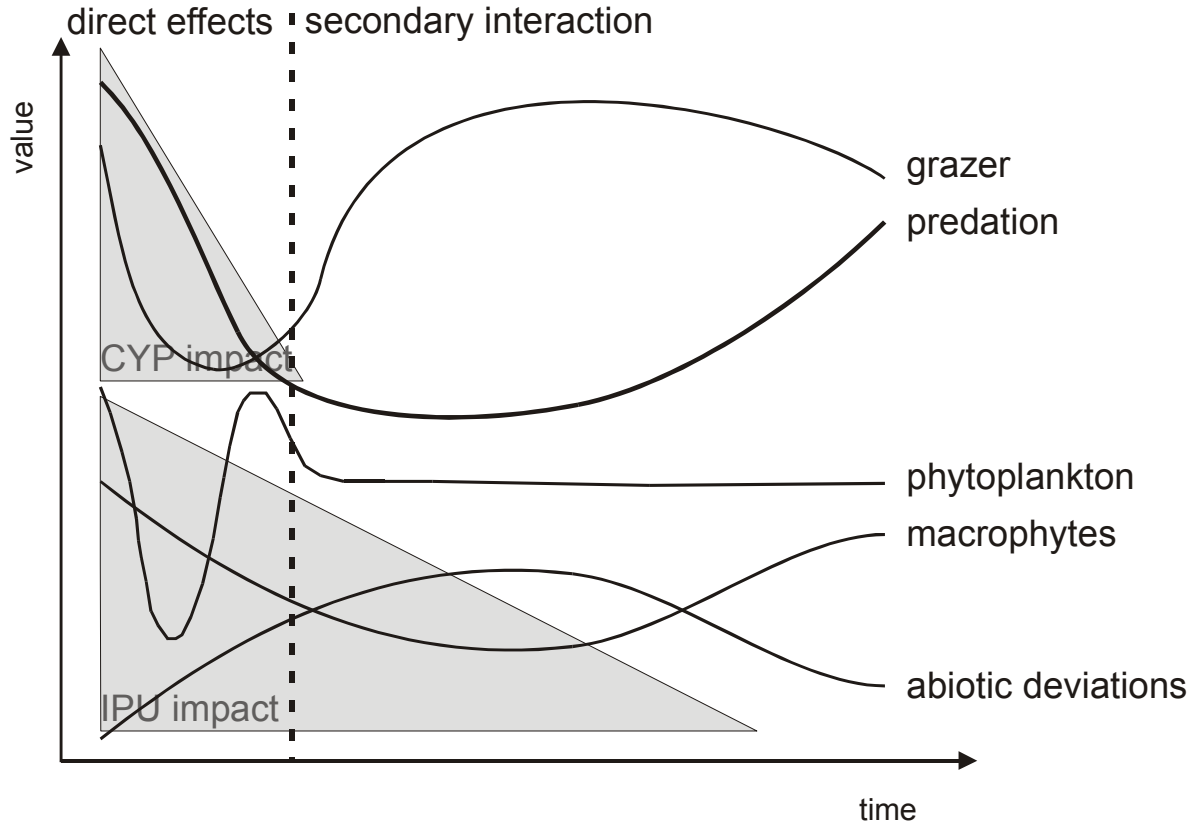


Figure 110: Ecosystem reaction on the combination treatment

Generalizing the findings noted above a general trend for the combined treatment can be deduced: The pesticides reduced susceptible organisms in the same way they did when applied separately (unless it was under strong top-down control, for example *Nephroselmis olivacea*). When an endpoint had direct links to another one *and* either of them was influenced by the treatment (e.g. *Cryptomonas ssp.* and grazers, *Chaoborus* and prey organisms), secondary interactions altered the system reaction specifically compared with the single substance studies. These effects occurred from that time on (dotted line), when the direct toxicity is lowered enough (via degradation of the active ingredients; grey triangles). These secondary interactions were more important when they were controlled top-down, although bottom-up effects played a role, too (see also the next part of this thesis).

Let me illustrate this with an example: Less predators (CYP) lead to more grazers which lead to less algae, i.e. top-down from first order consumers to primary producers through 3 trophic levels. This development is altered (somewhat balanced) by the process: Less primary producers (IPU)→more nutrients→more primary producers→more grazers (also three trophic levels, note the temporal component!). What actually has been seen was an increase in grazers, but no decrease in algae. A rationale for this is: There are three processes that influence the algae:

a) decrease due to grazing;
b) decrease due to the herbicide;
c) increase due to more nutrients.

When effect c) became important, effect b) is not present any more (or highly reduced), because the herbicide is degraded already. Effect a) also took some time to manifest itself.

Please remember that the secondary interactions discussed here were visible only after the pesticides have declined to some extent. Consequently, at the same (late) point of time there were two opposed impacts on the algae: grazing and nutrients. The results of the presented study propose that better growth conditions for the algae were directly exploited by the grazers so that an increase is only seen in them. The close linkage between grazers and algae was proven by the results of the IPU study. A real increase in grazers was not seen with IPU alone (because predation was constant). In contrast, increased grazing due to a lesser predator abundance was observed in the CYP study (although not to the extent as in the combination).

I therefore conclude that secondary pesticide effects are more important when they are triggered from top-down because they

1. appear in the single substance study already,
2. bottom-up effects of the single substance approaches do not out-weigh the top down effects in the combination.

The bottom-up effects, however, in some way or the other "regulate or facilitate" the intensity of the top-down effect.

An unsolved problem is the fact that the lowest treatment level did not show the least deviations from the controls. Whether this is a regular combination effect or a mere chance result should be clarified by additional research.

6 Linking the single substance approaches to the combined study: Results and discussion

It is rather trivial that there are many parameters that influence the development of the plankton in the test systems. However, by a multivariate analysis (Canonical Correspondence Analysis, CCA), the importance of some of them for the development with time was estimated. Advice how to interpret the diagrams can be found in TER BRAAK and VERDONSCHOT 1995. The aim was to find out which of the pesticides had a stronger impact on the plankton community and whether changes in the environment, some of which caused by the treatment, had influences on the development of the plankton in the presented studies. Such correlations were deduced above already, but with this analysis they can be proven statistically.

The "usual" interpretation of the CCA diagrams assumes that the species abundance is distributed in an unimodal way on the environmental gradient (LEGENDRE and LEGENDRE 1998). Consequently, even secondary treatment effects, some of which very distinctly reacted in an unimodal way (e.g. the abundance of the Copepod *Eudiaptomus gracilis* shortly after the treatment in the CYP and the combined study), can be dealt with properly in this analysis. This is especially important because the more "toxicological" methods (PRC, NOEC, NEC) all assume a linear reaction with the treatment level.

Secondly, some of the results of the three parts of this thesis are discussed synoptically in order to derive some more general aspects of system reaction to the combined treatment.

Last but not least, a model approach for predicting combined toxicity derived from laboratory studies has been tested on the outdoor data sets provided by the presented work.

6.1 CCA Analysis

6.1.1 Phytoplankton

Data of the single application studies were entered in the same analysis. In Figure 111, data sets of the CYP and the IPU treated sets of enclosures as well as the controls were given separate envelopes to make interpretation more easy. Labels for the data points, i.e. treatment level and date are not printed for a clearer display.

There is a clear development with time (environmental variable "day"). The areas of the controls and the CYP treated enclosures are almost identical in the analysis of the single substance studies (top). This is indicating that the consequences of the insecticide treatment on the phytoplankton did not alter the community structure to the same extend as the IPU treatment did. The data points of the latter study for enclosures that deviate strongly from the controls return to the controls rather fast (not a long distance on the variable "day"). The variables "temperature" and "day" are opposed to each other. This is rather trivial, because water temperatures get lower later in the year, of course.

More interesting is the behavior of the pH and the oxygen. Via photosynthesis they are linked to the IPU action. The arrows show in the opposite direction of the IPU arrow, clearly indicating that these abiotic parameters are lowered when more IPU is present. This is one part of the observed DO-pH-alkalinity-conductivity syndrome (KERSTING and VAN DEN BRINK

1997). Strange enough at first sight, alkalinity and conductivity are not pointing in the same direction as IPU. In the results of the IPU study they were both increased with the treatment level. Here, in the CCA, the CYP enclosures are also entered in the analysis. Both alkalinity and conductivity were not influenced in the insecticide study. As a result, CCA arranges them orthogonally to IPU and at the same time pointing in the same quadrant as the "day". Effects on both parameters took some time to develop, so this behavior is no surprise any more.

The CYP arrow points in the same way the temperature does, i.e. opposed to the "day". Therefore the interpretation is possible that the insecticide treatment sets the development in the phytoplankton in a way back in time. The community rather resembles that of the controls earlier in the year. However, the impact is not too strong because the CYP arrow is the shortest one. The strongest impact (i.e. longest arrow) had the "day", temperature, and IPU.

Please note that the strong deviations from the controls are solely triggered by IPU.

With regard to the combined treatment (bottom of Figure 111) the disturbance of the phytoplankton community is much stronger than in the single substance studies (more data points outside the envelope of the controls). This outcome reflects the fact that effects in the combination were generally more pronounced. The DO-pH-alkalinity-conductivity syndrome is reflected more clearly this time although IPU is pointing to the second quadrant and not to the first as the abiotic parameters would suggest.

An annual development in the controls is seen again. The lower treated enclosures are in the "cloud" of the controls (labels not shown here) indicating that at least some treatment levels had no changes in the community structure. When labeling the "sites", even the highest treated enclosures get near or overlap with the control area at the end of the year (pointed end of "day"). Thus, the disturbance is leveled out by this time.

Please note that the arrows of the pesticides are

a) longer for CYP and
b) shorter for IPU.

On the one hand that means that the impact of CYP is stronger in the combination (same direction as in the single substance study), on the other hand that in the combination IPU action is not so important anymore. The environmental parameters are all more important for the development of the phytoplankton community than IPU. Please note that major changes in these parameters were triggered by the IPU treatment in the first place. Of course, such a link cannot be "understood" by the multivariate analysis. In any case, the combined application altered the impact of the pesticides compared to their separate use.

Concerning environmental parameters please note that alkalinity and conductivity lead to a broadening of the area covered by the "treated" data points. In the combination, they have a more important influence on the phytoplankton community than in the single substance studies.

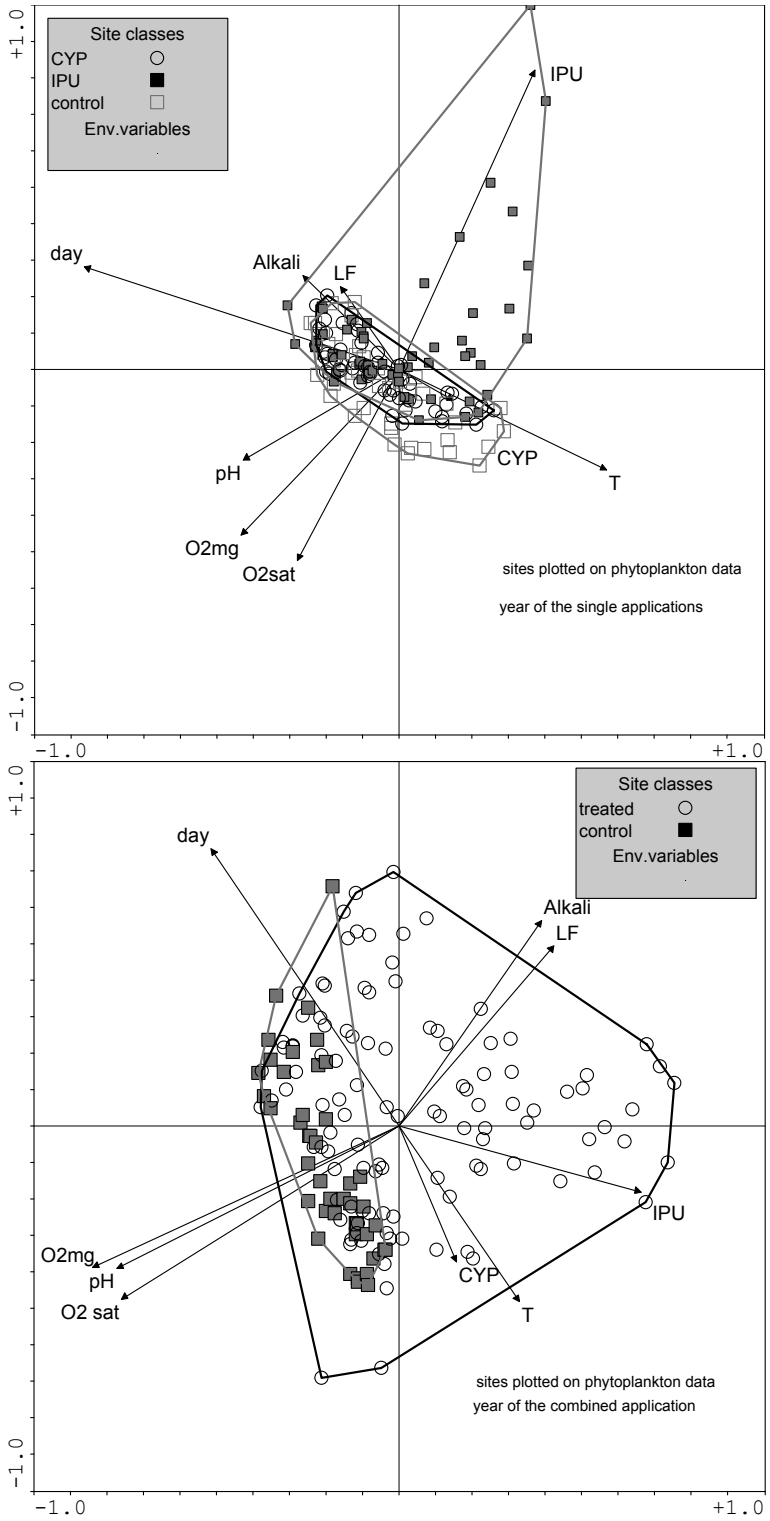


Figure 111: CCA on phytoplankton, data of the first year of the studies, top: single application, bottom: combination. --- day: sampling date, Alkali: alkalinity, LF: conductivity, IPU: Isoproturon, T: temperature, CYP: α-Cypermethrin, O2 sat: oxygen saturation, O2mg: dissolved oxygen

In short, what can be derived from CCA is:

1. Combined application leads to an altered impact of each pesticide compared to its single utilization,
2. The herbicide is more important for the changes in phytoplankton than the insecticide, even in the combination[34],
3. Environmental parameters have a greater influence on the phytoplankton community structure in a combined approach.

6.1.2 Zooplankton

Results of the CCA on zooplankton data are presented in Figure 112. Again, the single substance studies are on top of the diagram, and the combination at its bottom.

First of all, in the single substance study the time of the year ("day") outweighs all other parameters in its importance for the community development. Both pesticides have almost identical importance for their impact on the zooplankton. However, their arrows do not point to the same quadrant, i.e. the kind of influence is different. When looking at the "clouds" of the data points, the one for CYP is situated farther away from the controls. This finding indicates that the CYP enclosures where influenced more heavily than the IPU ones (compared to the controls). Deviations in the community structure are not too pronounced, though.

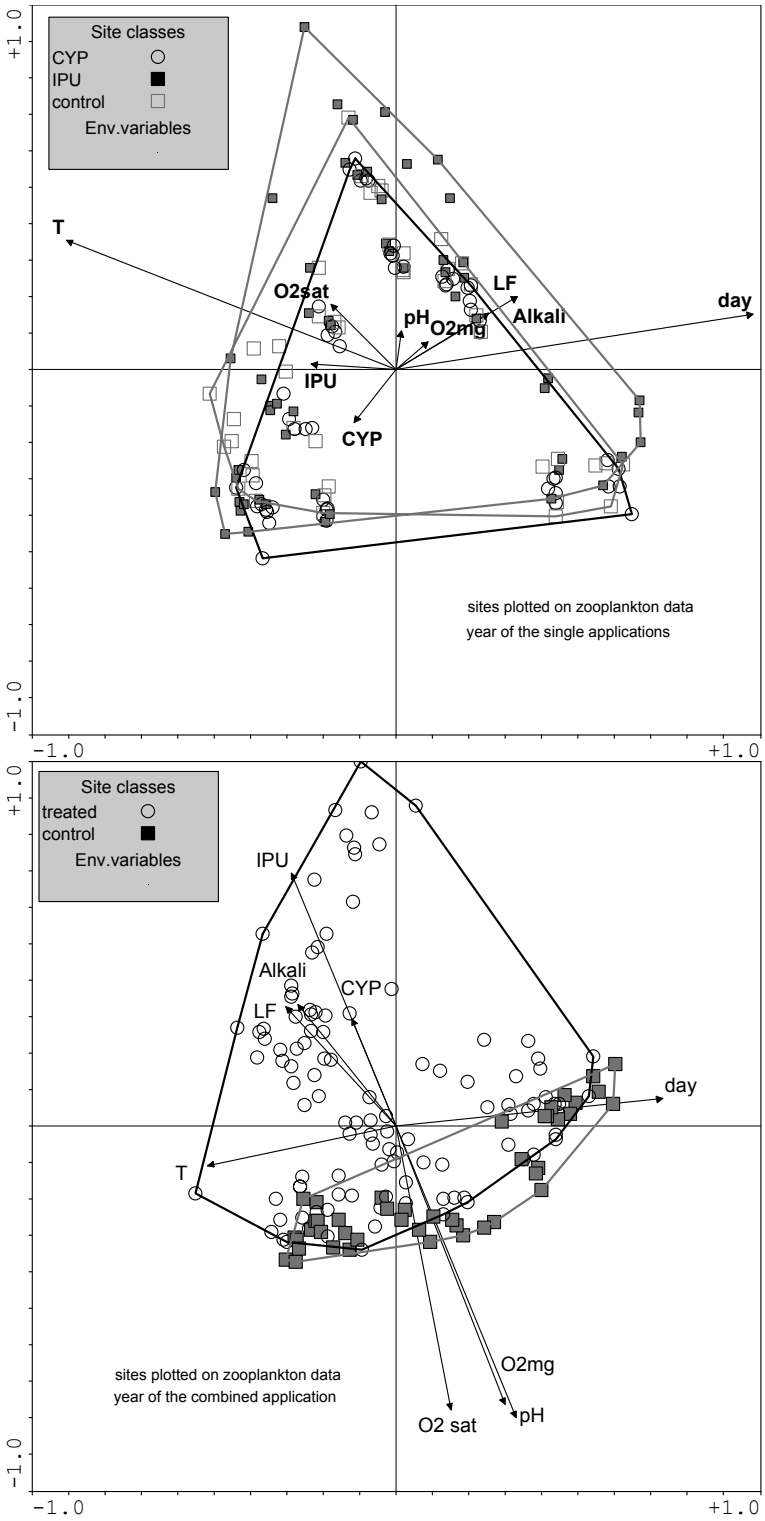
The picture is quite different when looking at the combination analysis. Here the controls build a band that indicates the development of the zooplankton community structure with time. The treated ones deviate heavily at first and get nearer the controls to the end of the year. Strange enough, for the DO-pH-alkalinity-conductivity syndrome arrows are the way they are supposed to be here but not in the algae, that are linked to this syndrome more directly via photosynthesis.

Both pesticides gain importance in the combination. They influence the zooplankton in exactly the same way, indicated by the fact that they lie over each other. IPU seems to have the stronger impact than the insecticide. The importance of the herbicide most probably stems from secondary interactions via the food web. Another reason may be that this is an artifact of the statistics. Please remember that in the phytoplankton IPU influence was less important than all environmental parameters in the combined study, but the changes in them were triggered by IPU, so its impact has in a way been under-estimated.

Environmental parameters gain importance in the combination application for the zooplankton community as well, again especially alkalinity and conductivity.

Conclusions drawn for the CCA on zooplankton are the analogous to the ones with the phytoplankton (see above, page 199).

[34] But please note that the influence of CYP has been higher than when used alone. There is no reason why CYP should be toxic to algae when combined with a herbicide and not if applied alone, so the stronger influence must be generated secondarily!



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6.2 Certain endpoints react differently in the combined approach

A general, over-simplified idea behind a possible combination toxicity can de formulated like this: A starving animal will not survive a stressor at concentrations that are as high as it can bear if it is well-fed. Daphnids may serve as an example: Lowering the abundance of the phytoplankton by the herbicide might, by the process given above, lead to a higher sensitivity of the Cladocera to the insecticide.

A process like this is too simple in many ways. For example, it does not take into account more complex food web interactions (both grazers and algae are influenced by other processes as well) nor the DT_{50} or the time it takes for toxic action of the pesticides to come into being, respectively. The most important intrabiocoenotic interactions are resource competition and predator-prey interactions (TILZER 2000). The combined treatment altered both control factors and therefore special effects of this treatment can be expected, because the single substances altered only one of the control factors (i.e. more effects from bottom-up or top-down).

A very important process is the modification of abiotic parameters that are particularly triggered by the herbicide treatment. HINDELANG 1993 was able to demonstrate a prolonged and more intense insecticide effect due to a lowered pH in a combination study with atrazine and Carbofuran. The lowered pH lead to a decreased decline of the insecticide.

As already demonstrated in the discussion of the combined study, a simple combination toxicity does not exist. For selected endpoints, combination effects are discussed in greater detail in the following. Please remember that whenever effects in the combination were similar to the single substance approaches, they had approximately the same NOEC but were more pronounced.

An overview of NOEC data is given in Table 63. The over-all NOEC is identical to the single substance approaches. Most NOECs in the combination were identical to the one of the single substance with the stronger impact. This means that there are no combination effects for the NOEC. However, it cannot be excluded that effect concentrations for the individual substances were lower in the combination although the NOEC is not changed (WALTER 2002, FAUST *et al.* 2003).

Whenever NOECs are higher in the combination a release from the top-down control was the reason (especially in the algae). A lowered NOEC was observed in *Chroomonas acuta*, and the Chrysophyceae. For the first one, NOEC of IPU may be too high for statistical reasons (NEC is lower, see the combination and the IPU part). Consequently, no real differences may be noted. The Chrysophyceae were able to take advantage of the release from the top-down control (reduced grazing due to CYP action). The lower NOEC here thus stands for an increase in this algal class.

Table 63: Overview of NOEC data

taxon	NOEC [µg/L]CYP	NOEC [µg/L] IPU	NOEC [µg/L] CYP/IPU
Water quality parameters	n.n.	4	0.075/16
Macrophytes	n.n.	64	0.075/16
Chlorophyceae	0.015	64	0.075/16
Chroomonas acuta (Cryptophyceae)	0.375	16	0.015/4
Chrysophyceae	0.015 for decrease	16	0.015/4
Cryptomonas erosa et ovata (cyrptophyceae)	0.015	16 (the same for both years)	0.075/16
Cyanophyceae	0.75	n.n.	0.375/64
Desmarella moniliformis (Chrysophyceae)	0.075	no pronounced effects	n.n.
Monosiga varians (Chrysophyceae)	0.375	128	n.n.
Nephroselmis olivacea (Chlorophyceae)	0.375	4 or lower	n.n.
total abundance (phytoplankton)	0.075	not clear, maybe between 16 and 64	0.075/16
$NOEC_{community,\ phytoplankton}$	0.075	16	0.075/16
*Chaoborus crystallinu*s (Insecta)	<0.015	no effects at all	< 0.015/4
Chydorus sphaericus (Cladocera)	0.75	4	0.075/16
Cladocera	0.075	16 (days 3-14 a.t.)	0.075/16
Cyclopoida (Copepoda)	0.75	≤ 128 (increase in autumn)	< 0.015/4 (does not apply)
Eudiaptomus gracilis (Copepoda)	0.015	n.n.	0.375/64 (does not apply)
Nauplia ssp. (Copepoda)	0.015	<4	0.015/4
Rotifera	0.750	16	almost unaffected
Simocephalus vetulus (Cladocera)	0.075	4	0.075/16
total abundance(zooplankton)	0.015	4	0.075/16
$NOEC_{community,\ zooplankton}$	<0.015	64	0.075/16
Over-all NOEC	**<0.015**	**4**	**<0.015/4**

6.2.1 Water quality parameters and pesticide residues

Effects of the combined study were identical to the herbicide study (with respect of the mode of action). In both cases, a DO-pH-alkalinity-conductivity syndrome (KERSTING and VAN DEN BRINK 1997) was found. NOEC was higher in the combination study due to less or different impact of IPU on the algae. Variations stemmed mainly from a decreased impact of IPU on phytoplankton algae due to simultaneously lowered grazing pressure (cf. 6.2.2 and the Combination part).

In any case, the pH of the enclosure water was lowered by the herbicide in the combined approach. Data from the literature (PERKOW 1988) indicate that CYP is more stable in neutral or acid waters and more readily degraded in an alkaline environment. However, a prolonged DT_{50} or more intense direct CYP effects on zooplankton could not be demonstrated here. The latter finding is in line with results presented by FAIRCHILD *et al.* 1994. They found no increased sensitivity of zooplankton organisms towards Esfenvalerate (another pyrethroid insecticide) when it was applied in combination with atrazine. For details of the impact on zooplankton in the combination used in this study please refer to 6.2.3 and the combination part (5.6).

6.2.2 Phytoplankton

Both single substance studies revealed impact on the phytoplankton. Effects in the combined approach had an intermediate state.

With the insecticide alone, right after the application more algae are present in the test systems. Decreased grazing pressure is the reason for this. In the long run, the loss of the main predator, *Chaoborus crystallinus*, leads to an increase in grazers and consequently less algae (at least in the higher concentrations).

In the IPU study, sensitive algae like *Chroomonas acuta* suffered a loss in abundance due to the herbicide.

Effects in the combination study for this taxon was comparable with a slightly lower NOEC towards IPU. It is concluded that if a species is quite sensitive towards a stressor, combined effects of toxicant mixtures are, if at all, of minor importance (cf. also 6.2.3a).

Another very IPU sensitive taxon was *Nephroselmis olivacea*. In the combined treatment, no effects were visible. This species is a very small green algae and has a reproduction pattern that flows an “r-strategy”. Consequently, the release from the top-down control enabled it to compensate for the losses due to the herbicide completely. It therefore leads over to the next group of taxa.

More robust ones showing only minor toxic effects in the IPU study were additionally affected secondarily in both the IPU and the combination study. The main nutrition for zooplankton grazers are the *Cryptomonas ssp.* (cf. INFANTE 1973, AHLGREN 1990, KIRK 1997), which abundance is closely linked to *Simocephalus vetulus* in particular, the most abundant big Cladoceran in the test system. Due to an initial decrease in the algae several parameters enter a oscillating pattern in the IPU approach that clearly demonstrates the disturbance of the whole test system and not only the sensitive species.

In the combination, such oscillations were leveled out. Appling IPU together with CYP altered the consequences of the IPU impact on the algae. Abundance of phytoplankton decreased treatment related in the first week, and increased later on due to less grazing (CYP action). For the remaining time no strong variations were visible. The combination treatment therefore clearly indicated ecosystem functioning: The link between grazers and nutrition was not broken but the effects added up in a way.

Contrastingly, the indirect effect of CYP (increase) on the Chrysophyceae was amplified in the combination treatment. This was also reflected in the lower NOEC of this class. Top-down control is therefore an important factor for this group of algae.

6.2.3 Zooplankton

6.2.3a *Some consequences of abundance changes in* Chaoborus crystallinus

The planktonic midge larvae are a keystone species for the test systems used, because no fish are present. By this lack, they become the most important predator that regulate plankton abundance from "top-down". Additionally, they are most sensitive towards CYP, but IPU is not at all toxic for them. In this species no combination effect could be demonstrated. The high susceptibility over-compensated any (in this case only secondary) different effects.

As a result, the food web reacts differently in the combination than in the single substance approaches. Secondary effects on grazers, e.g. *Simocephalus vetulus* or *Eudiaptomus gracilis*, that have been controlled either predominantly top-down (CYP, less predation) or bottom-up (IPU, less algae) are coming from both sides in the combined approach.

Such effects may level out: Less algae lead to less grazers but less predation leads to more grazers. However, such a balance is very improbable. Indeed, the effect of the top-down control is stronger than the bottom up one in the presented study: The most prominent example for this behavior are the Copepods. *Eudiaptomus gracilis* will be discussed in greater detail below (6.2.3c), Cyclopoids and Nauplii are dealt with in the following.

Results of the single substance treatments revealed no or only very slight effects on the Cyclopoids. In the combination, these organisms are affected quite heavily. In the long run, an increase was seen. The same holds true for the Nauplii, a finding that is rather trivial because more adults are leading to more offspring. Since no increase could be demonstrated in the CYP study, the top down control alone is not able to facilitate changes in the abundance. The same holds true for IPU: no (bottom-up controlled) decrease has been seen there[35]. In the combination, only the effect of the top-down control is visible, thus demonstrating its superiority over the bottom-up one. However, the bottom-up changes are needed to alter competition in zooplankton so that the increase can come into being.

6.2.3b *Strong secondary interactions with* Simocephalus vetulus

Two different impacts were observed on this Cladoceran: Strong secondary interaction with the algae in the IPU study (oscillations) and sensitivity towards CYP. Of course, the insecticide plays the more important role in the first weeks when both pesticides are present in the system. Secondary increases due to less predation are, again, more pronounced in the combined approach. The oscillations with the phytoplankton are not allowed to build up, corroborating the interpretation that the top-down processes (i.e. for example predation pressure) are of greater importance for the plankton regulation in the presented study.

6.2.3c *Shifts in the reaction of* Eudiaptomus gracilis

This calanoid Copepod shows a very interesting behavior towards the three treatments (Figure 113). In the controls, it is regularly found but with a very low abundance. The IPU treatment did not lead to higher, significant increases. In both the CYP and the combined

[35] This assumes that the Cyclopoids either feed on the algae directly or that less algae lead to less prey for the carnivore copepods.

approach such increases could be demonstrated. Consequently, these increases must be triggered by the reduced predation of *Chaoborus crystallinus* (cf. PASTOROK 1980 (in LIAR 1990). The major difference is the position of the maximums in Figure 113.

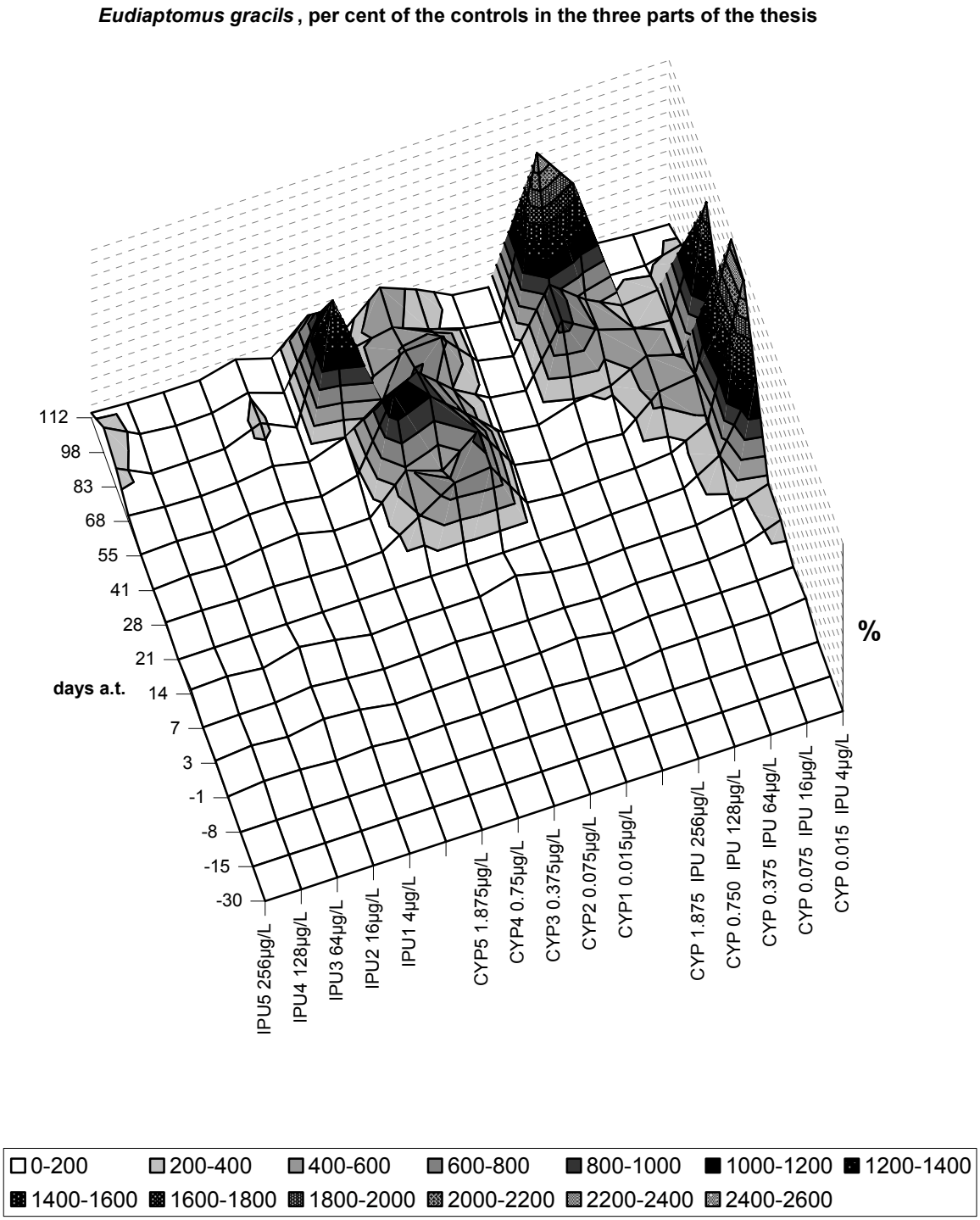

Figure 113: Per cent of the controls of *Eudiaptomus gracilis* under the three treatment regimes

With CYP alone, two peaks are visible: On day 55 a.t. in CYP4 and day 83to 98 in CYP5. In the combination, the maximum in level 5 on day 83 to 98 a.t. is stretched to level 4 as well. The highest deviations shift to the later date.

More interesting is the long lasting increase in level 1 (day 21 to 68 a.t.). Thus, the lower maximum starts at lower concentrations of CYP and lasts longer than with CYP alone. So what is an explanation for this reaction?

Clearly, CYP treatment is needed to enable any increase in the first place. More *Eu. gracilis* are found on day 21 in the earliest case, i.e. when most (or all) CYP residues are depleted from the water column. Other grazers (i.e. competitors) start to reach control levels at this point of time already. As demonstrated by the biomonitoring, the single species tests, and the CYP study, *Eu. gracilis* is sensitive towards CYP, although it is less susceptible than *S. vetulus*, for example.

Consequently, there are three processes that facilitate the increase in the Copepod in the CYP study:

1. lower sensitivity to CYP than competitors;
2. depletion of the insecticide;
3. less predation.

With IPU additionally in the water in the combined approach, please remember that *Eu. gracilis* is tolerant towards the herbicide even at higher concentrations than in level 5. In the IPU study, there are two small peaks. Although the counts are between 200% and 400% higher than in the controls, the per cent values themselves are of minor relevance because the controls have only very low counts (mostly below 10/L). These peaks appear late in the year (day 83 to 98 a.t.) in IPU1 and IPU5. They are hinting at some promoting effect of the IPU treatment, although this cannot be proven statistically.

In any case, they coincide with the maximums in the combined study with respect to the treatment level. Indeed these maximums are higher in the combination than with CYP alone, where peaks were also seen. Consequently, this must be regarded as a combination effect. The fact that the increase in level 1 starts so much earlier than the slight peak in IPU1 does not hinder this interpretation, because food web interactions may well be delayed in the herbicide study. The combined approach enables the effect to come into being quite early and pronounced. The maximum in level 5 is boosted by the IPU effect as well.

In short, an effect that is negligible or insignificant in a single substance study may well lead to major deviations when another stressor is present in the test system. Such a finding was also seen in laboratory studies of WALTER 2002 and FAUST *et al.* 2003.

6.2.4 Conclusions

In the presented combination study the two pesticides were chosen to have no overlap in their toxic action. Pesticide residue analysis found no different degradation due to changes in the abiotic environment. Therefore, species that are highly sensitive to one of the pesticides react in the same way as they do in the single application studies unless they are not strongly controlled from top-down additionally. Taxa with an intermediate susceptibility are influenced strongly by the pesticide mixture via food web interactions. These interactions can lead to boosts in unimportant effects with only one stressor. Taxa that show no effect to either pesticide can well react to their combination, again via the food web. The top-down control was of greater importance for all the (secondary) effects in the combination than the bottom-up one.

The taxa richness in the zooplankton was stronger negatively affected in the combined treatment study (but not the one of the phytoplankton). This can be explained by the alterations in the biocoenotical control factors again: zooplankton receives negative impacts from bottom-up (IPU sensitive algae decrease or are of poorer food quality) *and* due to the direct CYP action on them (less grazing or death). The reduced predation pressure cannot counter-balance this development, because the test ponds are isolated from other natural waters, so the immigration of extinguished species is hindered to some extend. However, the loss of rare species in the zooplankton cannot be excluded even under natural conditions.

Together with the more intense effects on moderately susceptible taxa that are facilitated by insignificant impacts in the single substance study, both these findings are strong arguments against the concept of the NOEC (cf. 7).

In sum, predicting combination effects is quite difficult. Well-known food web interaction are urgently needed. However, two promising mathematical models have been established using laboratory tests without regarding such interactions; one of them was used on the data of this thesis. The results are presented in the following.

6.3 Predicting combination toxicity for single substance data: A model approach

6.3.1 The model: BLISS independence (response addition, independent action (IA))

There is an urgent need for a model that is able to predict mixture toxicity from data of single substances (e.g. VIGHI 2003, FAUST *et al.* 2003). Effect concentration for single substances are provided by a regular basis by now and are indeed part of the framework the EU requires to permit a pesticide on the market (cf. EU Council directive 91/414/EEC, BBA 1998). At least in waters near agricultural areas, mixtures of several pesticides were found regularly (e.g. KREUGER and TÖRNQVIST 1998, HÖCKER and NEGELE unpublished, HOUSE *et al.* 1997, GARAMOUMA 1998). Effects of such mixtures cannot all be tested separately because there are simply too many substances available. Therefore, a model that can calculate mixture toxicity is demanded. In laboratory studies, two models were found to be able to predict such mixture toxicities quite nicely, the *concentration addition* (CA or LOEWE addition) and the BLISS independence (response addition, independent action, IA), (cf. ALTENBURGER *et al.* 1996, FAUST *et al.* 1993, WALTER 2002, FAUST *et al.* 2003, VIGHI *et al.* 2003). The latter model was used here. Reasons for this choice are given in the following.

The model used here was developed by BLISS 1939 and GRECO *et al.* 1995. Its mathematical form was derived from BERENBAUM 1985. The mixture toxicity that this model calculates for two toxins can be described like this: A certain percentage of individuals of a certain taxon is killed by agent A. Those surviving this “attack” are killed up to another percentage by agent B (WALTER 2002). In this way, the model not simply adds up certain effects like two agents were

complementary in their action on a certain organism/population etc., but observes their mixture toxicity as an independent combination of both their effects[36].

It is not as widely regarded as a "general" model for mixture toxicity as the *concentration addition* (LOEWE addition, cf. ALTENBURGER *et al.* 1996, FAUST *et al.* 1994, WALTER 2002, GRECO *et al.* 1995). First of all, the model has the premise that the substances in the mixture have an independent mode of action (on a molecular basis). Whether or not this independence is preserved throughout all the complex interactions in a biological system until a certain effect (i.e. death of an organism) comes into being has been discussed before (e.g. BERENBAUM 1985, ALTENBURGER *et al.* 1993 in ALTENBURGER *et al.* 1996, WALTER 2002). ALTENBURGER *et al.* 1996 concluded that the BLISS independence rather underestimates mixture toxicity in laboratory studies. Contrastingly, WALTER 2002 found the BLISS independence the better prognostic tool for mixture toxicity for mixtures with an unknown mode of action. Other, more recent studies point out the importance of the mode of action (FAUST *et al.* 2003, VIGHI 2003). VIGHI *et al.* 2003 found that the *concentration addition* is the better tool when predicting mixture toxicity on multi-species test systems, but they also pointed out that the substances used in their study were all phenylurea with comparable modes of action. FAUST *et al.* 2003 conducted a study with 16 substances with strictly different modes of action. They concluded that the BLISS independence was the more accurate tools than the *concentration addition* that rather over-estimated combination toxicity for these substances. The molecular mode of action is therefore an important criterion when choosing the model for predicting mixture toxicity.

An advantage of the BLISS independence is that it can also be used for concentrations below the NOEC of the substances in the mixture (WALTER 2002, FAUST *et al.* 2003). In the study of WALTER 2002, he was able to demonstrate that a combination of toxins below their individual NOEC (in the combination) actually had effects on certain endpoints. Comparable results[37] were seen here in this study in the Cyclopoids, for example. WALTER 2002 therefore regarded the concept of a NOEC as at least questionable for toxicant mixtures. Other problems with the NOEC concept are summarized in VIGHI *et al.* 2003. In the presented study, NOECs of the combination were not generally below those of the single substance studies, merely the effects of the toxic action (direct or indirect) were more severe.

Another advantage of the BLISS independence is that it readily fits in the regression design that is advisable for ecotoxicological outdoor studies (e.g. EU 2002, CLASSIC 2001, HARAP 1999). By such a regression design, EC_x values of the treatment levels used can readily be calculated and then used in the model (like it was done in the presented study). When calculating mixture toxicity with the *concentration addition* model by ALTENBURGER *et al.* 1996, there is the need for the concentration (single substances and their combination with a fixed ratio of the substances) with a specific EC_x (for example, the EC_{20} of each pesticides for a certain taxon), because combination toxicity can only be derived from such data by this model (BERENBAUM 1985, WALTER 2002). Such a concept may be worked out in the laboratory, but it

[36] Supposed both agents kill 50% of the population, then BLISS independence predicts a combined action of 75% killed: 50% by agent A *plus* 50% of the remaining 50%, i.e. 25% by agent B.

[37] i.e. that they are not following the concept that a combination of the substances at their NOEC leads to no effect even in the combination.

cannot be used in standard outdoor experiments, because of the tremendous (and expensive) effort that would have to be taken for this[38]. The number of repeats and the number of concentration steps must be higher because of statistical needs when an exact regression should be possible (cf. MAISE 2002). Imagine, for example, only determining the EC_{20} for the most important zooplankton species alone: *Simocephalus vetulus*, the Cyclopoids, *Chaoborus crystallinus*, the Rotifers, and so on for the single substances and their combination.

In short, the BLISS independence is the concept that better fits with outdoor experiments: Determining the percentage of individuals of a population that are affected in a certain treatment level is always possible, (almost) regardless of the number of repeats or treatment levels. In the presented approach, those C_x/D_x values were integrated where a trend with the concentration was visible at least with one pesticide. Please note that this is not equal to a regular linear regression. Values rising (or falling) with the concentration was the only premise taken. However, such an approach disregards endpoints that have an unimodal reaction to the treatment. On the one hand, only direct toxicity and simple secondary effects[39] can therefore be predicted by the model. On the other hand, unimodal effects were predominately observed in the combination. The algorithm of the BLISS independence is not capable of predicting effects that occur exclusively when substances are applied together. It was designed to combine the effects of these substances but it cannot anticipate completely new impacts that occur via food web interactions. Consequently, restricting the analysis of prediction quality (IPQ index, see below) to effects that can actually be calculated by the model is justified[40].

The pesticides used in this thesis were chosen not to interact on a molecular basis, so the premise of the BLISS model is met. So calculating mixture toxicity with this model is valid for the data generated here. In order to have an idea whether the model under- or over-estimates the mixture effects, the IPQ (Index of Prediction Quality, GRIMME *et al.* 1994, ALTENBURGER 1996) was calculated. Please note that values below zero are an overestimation of the effect by the model (i.e. the combination has actually less effect than predicted). Values higher than zero indicate an underestimation of the combination effect. IPQ values are a factor by which the predicted effect of a certain treatment level is deviating from the actually observed one. In the following, "effect" for taxa data always means the determined or the predicted C_x at a certain treatment level for the endpoint in question. For water quality parameters, it denotes the D_x of the treatment level.

[38] Contrastingly, EC_{50} data for single species for many substances is readily available from data in the literature. As a result, FAUST *et al.* 2003 suppose less problems for calculating a predicted mixture toxicity with the CA model. This good data base is not available for most outdoor studies and their evaluated endpoints. Here additional research has to be done and thus requirements for the IA may be met with less effort.

[39] For example less grazing due to reduction of zooplankton by the insecticide that is leading to more algae (without other influences on both endpoints).

[40] You will not use a hammer if you want a screw removed and then say that a hammer is poor tool…

6.3.2 Prediction quality

6.3.2a Water quality parameters

This is the simpler case for predicting combination effects. The insecticide treatment had no effects on the water quality, so all effects in the combination are to be derived for the herbicide action.

However, these alterations are of secondary nature: IPU is influencing the algae and the macrophytes and by their reaction (less photosynthesis) impact on the water quality occurs. Since there was a steady trend in the IPU study, IPQ values were calculated (rationale see above, 6.3.1). In the combination, some secondary/tertiary effects (at least in level 1) were discussed (see above). The question is now whether the BLISS independence can predict changes in water quality accurately even under these circumstances.

Please remember that effects were almost identical to the IPU study, so accuracy is supposed to be high. The results are presented in Table 64.

Table 64: IPQ values for water quality parameters

IPQ	Valid N	Mean	lower Quartile	upper Quartile	Minimum	Maximum	Range	Variance	Std.Dev.
pH	81	1.1	-1.7	3.4	-26.1	32.8	58.8	60.1	7.8
O2 saturation	70	-0.2	-0.2	0.1	-2.2	0.4	2.6	0.3	0.5
Conductivity	95	0.0	-0.2	0.0	-0.5	0.9	1.4	0.1	0.2
Alkalinity	50	-0.1	-0.2	0.0	-0.6	0.7	1.3	0.0	0.2

Excluding the pH, prediction quality is very good. The highest deviation is seen for the oxygen saturation with a factor of –2.2. This is in the range of laboratory studies with data of single species (ALTENBURGER 1996, WALTER 2002). Consequently, the BLISS independence was able to predict the secondary action of the combination on the three parameters oxygen saturation, conductivity, and alkalinity.

For the pH, the mean (factor 1.1) and the quartiles are acceptable. Minimum and maximum values rather indicate poor prediction quality. The model was therefore able to foretell the alterations in the pH only on a general level, but it is not specific enough to precisely predict changes when looking at special treatment levels. A reason for this poor quality may be the strong inversed relation of level 1 and 2 to the combined treatment. Please remember that level 1 had lower pH than level 2 in the most of the time. A rationale has been given in the combination part of the study. If any, this inversion is the kind of "unpredictable" combination effect discussed in 6.3.1 (due to food web interaction). As already noted above, the model cannot be blamed for not being able to predict such an effect. Impact of the combined treatment at higher concentration levels was indeed forecast quite well.

To sum it up, the BLISS independence was rather apt to predict combination treatment effects on water quality parameters.

6.3.2b Plankton data

A general overview of the IPQ values for zoo- and phytoplankton is given in Figure 114.

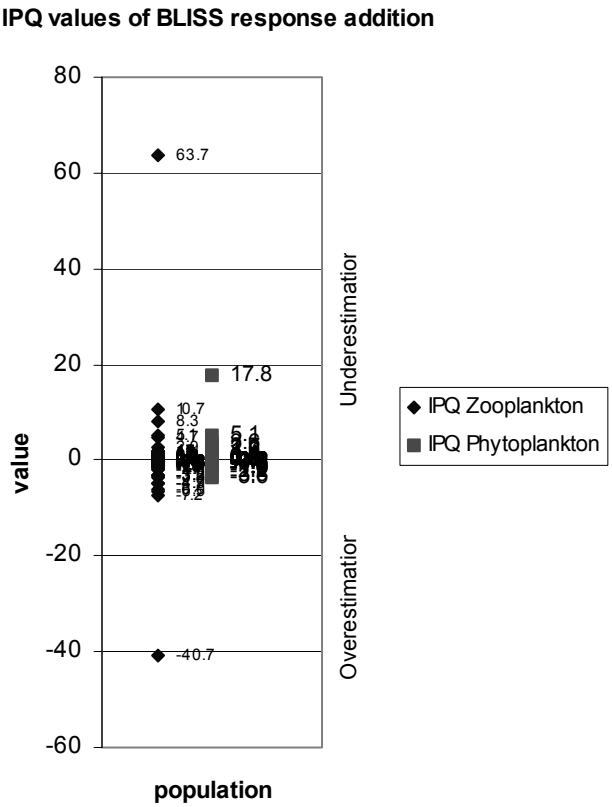

Figure 114: IPQ values for BLISS response addition

Effects for the phytoplankton are predicted better than for the zooplankton. All values are between factor –20 and +20. The majority of all data points (zooplankton included) is inside –10 and +10. Deviations in data derived from laboratory studies were not higher than factor 2 to 4 (ALTENBURGER 1996, WALTER 2002), but please note that the prediction made here were derived from an outdoor experiment. Higher variability can be expected, because the environment for the organisms cannot be controlled as much as in an indoor experiment. Thus, it is viable that the error in the prediction is about three to five times higher than for laboratory experiments. Moreover, data for these factors were derived by multi-species analysis in the presented work and not by only one species as in the literature cited.

When looking at less summarized data, factors are getting better (Figure 115, Table 65). For all phytoplankton taxa, 50% of the IPQ values are deviating only by a factor <2.

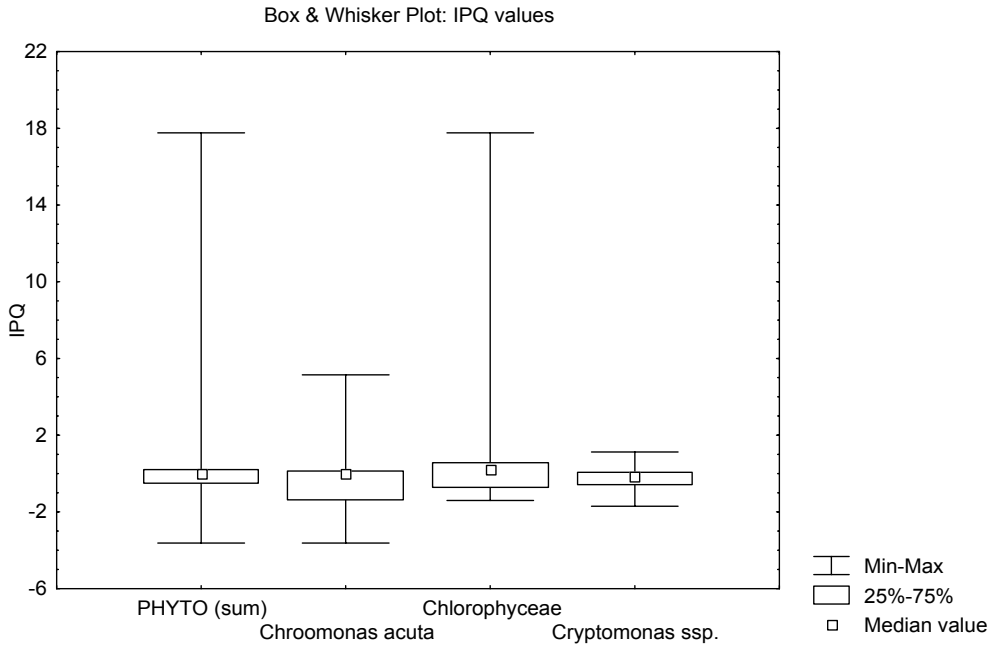

Figure 115: Box and whisker plots of the IPQ for selected phytoplankton taxa

Taking a closer look at the data in Table 65, the means of the factors are all smaller than 1 except for the Chlorophyceae and the Nauplii. The data of the quartiles indicate that the prediction quality of the BLISS independence is quite fine; 50% of all predictions are better than factor 3, most of them even better than factor 1.

Even the factors for heavily secondarily affected taxa (*Cryptomonas ssp.*, *Eudiaptomus gracilis*) are well inside this range. In particular, the clear combined impact of IPU and CYP on *Eu. gracilis* was predicted with great accuracy. All absolute values for the IPQ were smaller than 2 between day 28 a.t. and 55 a.t..

To sum it up, the BLISS independence model for the prediction of toxicant mixtures works fine with the data of the outdoor studies if a steady trend is seen in the reaction to the single substance applications. The combination of the pesticides met the premise of the model that the mode of action an the molecular basis is dissimilar.

Table 65: IPQ data for some taxa

IPQ	Valid N	Mean	lower Quartile	upper Quartile	Minimum	Maximum	Range	Variance	Std.Dev.
Phytoplankton (sum)	59	0.2	-0.5	0.2	-3.6	17.8	21.4	7.3	2.7
Chroomonas acuta	31	-0.1	-1.4	0.1	-3.6	5.1	8.8	3.0	1.7
Chlorophyceae	9	2.0	-0.8	0.5	-1.4	17.8	19.2	35.9	6.0
Cryptomonas ssp.	16	-0.2	-0.6	0.0	-1.7	1.1	2.8	0.4	0.6
Zooplankton (sum)	155	0.1	-0.5	0.2	-40.7	63.7	104.5	40.6	6.4
Nauplii	17	-3.1	-2.9	0.4	-40.7	1.3	42.0	99.2	10.0
Eudiaptomus gracilis	26	-0.2	-0.9	0.0	-6.0	1.7	7.6	2.1	1.4
Rotifers	19	0.7	-0.5	0.9	-1.4	10.7	12.1	6.5	2.6
Cladocera	9	-0.9	-2.5	0.1	-3.8	1.3	5.0	2.7	1.7
Chaoborus crystallinus	53	0.0	-0.2	0.0	-7.2	5.1	12.3	2.4	1.5
Simocephalus vetulus	18	-0.1	-0.2	0.0	-3.3	1.7	4.9	0.8	0.9

7 Final conclusions and perspectives

The presented work gave deeper insight in four major questions:

1. What are the effects of CYP and IPU treatment on the plankton of an aquatic mesocosm?
2. In which way are these effects conveyed through the food web and through time?
3. What are the differences in these results when the two pesticides are applied jointly?
4. Can these differences be predicted and/or can a pattern for the system reaction be derived for combination effects?

A question that arises regularly when discussing the outcome of outdoor mesocosm data is whether the results are reliable between different study years or laboratories. Data for CYP derived from three studies in three different years at the FG Ökotoxikologie resulted in almost identical conclusions for several endpoints (data unpublished). When comparing the data of the IPU part to the outcome of the study of ESER 2001, results were consistent, too. Moreover, GIDDINGS *et al.* 2001 found ecotoxicological data for pyrethroids remarkably constant for seven different mesocosm studies on two continents over a whole decade. To sum it up, comparing the results between the years and the mesocosms is certainly viable.

In the CYP study, all effects on the biocoenosis triggered by the treatment are related to the sensitivity of organisms that are situated on a higher trophic level. The sensitivity towards the agent increased with the trophic level (i.e. the top predator is at the same time the most sensitive organism).

Depending on the amount of insecticide in the water effects were conveyed secondarily and tertiary through the food web, depending on the susceptibility of the organisms on the higher trophic level and their role in the food web. The higher an organism was located in the food chain, the wider its effect were transmitted to organisms on a lower position. Sublethal effects (reduced grazing) also played a part in this scenario by shifting the ecotoxicological parameters of an endpoint to a higher sensitivity than would be expected by lethal effects alone. Impacts were therefore clearly controlled from top-down.

The temporal component did not play a major part for the effects. Depending on the life cycle of the organism impact was leveled out.

The main reason for this clear-cut top-down effect cascade was that the abiotic environment and the structural features (i.e. macrophyte density) had not been altered by the treatment. A model is given in Figure 35 on page 76.

In the IPU study, two scenarios had to be differentiated: the first one where macrophytes were not affected and the second one where they were reduced by the treatment. However, phytoplankton species that were very susceptible to the herbicide were not influenced in this way. They were simply decreased in relation to the treatment and recovered accordingly.

In the first case (macrophytes were not affected), abiotic parameters were not changed dramatically. Influences were transmitted through the food web bottom-up, because the algae had been affected directly. Here again sublethal effects (poorer food quality) were able to play

a part. The macrophytes stabilized the whole system in a way that interactions did not cause too strong effects.

In the second case, the link between grazers and algae became very evident. Almost all endpoints that were affected in one way or the other showed recovery not before the macrophytes themselves began to recover. For many parameters, an oscillating pattern was observed that was correlated to the abundance of the dominant algal taxa. The amplitude of these oscillations decreased with the abiotic changes being leveled out due to the development in the macrophytes. Again, the control was exerted from bottom-up.

Both scenarios were modeled graphically in Figure 74 on page 125.

In the combination treatment, the factor time became even more important. The DT_{50} value of the pesticides determined whether direct or indirect effects were of greater importance. As long as a direct CYP impact was present in the system, the (direct) toxic and the more simple secondary interactions prevailed. For example, grazing pressure was reduced (direct effect of CYP). Thus, IPU resistant algae like the Chrysophyceae or less sensitive one like the Chlorophyceae increased. Later one, more complex food web interaction that were predominantely controlled from top-down (and only facilitated from bottom-up) were observed.

Structural deviations due to the IPU impact on the macrophytes did not differentiate the reaction of some endpoints in two groups like in the IPU study. The time-depending combined action prevented oscillations from building up. Top-down effects outweighed bottom-up ones. A model for the system reaction to the combination treatment is given in Figure 110 on page 194.

Special combination effects were mostly controlled top-down, e.g. the increase in Chrysophyceae or Cyclopoids. In *Eu. gracilis*, insignificant effects of the IPU treatment lead to a boost in the effect of CYP in the combination.

Very important for risk assessment implications is the finding that the species richness in the zooplankton was affected much more strongly in the combination approach. An effect on the overall species richness or density is referred to as “unacceptable” in EU 2002, for example. Thus, protecting species richness and/or keystone organisms (which are important aims of protection, cf. HARAP 1998, EU 2002) may at least be complicated (if not impossible) by assessing single substance data alone.

The latter two findings (amplification of insignificant effects, impact on species richness) provide strong arguments against the concept of the safety of the NOEC of plant protection products. In the risk assessment procedures suggested by the EU (EU 2002), one key objective of the regulative procedures is the protection of the environment against side-effects of plant protection products that are regarded unacceptable. The concept of the NOEC, that was already challenged by WALTER 2002, FAUST *et al.* 2003, BACKHAUS *et al.* 2000, FAUST *et al.* 2001, or HANSON and SOLOMON 2002, again proved to be unsafe when active ingredients enter an environmental compartment jointly. As demonstrated here, even totally independently acting toxic agents can have unforeseen effects at concentrations that must be regarded safe by the NOEC concept (for an endpoint in question). Unfortunately, mostly combinations of pesticides are found in natural waters (KREUGER and TÖRNQVIST 1998, HÖCKER and NEGELE

unpublished, HOUSE *et al.* 1997, GARAMOUMA *et al.* 1998, NEAL *et al.* 2000). In this context the reaction of the Cyclopoids to the combined treatment must not be forgotten. They were not affected even at the second highest treatment levels in both single substance studies, but showed a totally different, "J-shaped" type of reaction to the combination treatment. This effect was a secondary one (via the food web) and by no means expected from the results of the single substance studies. This endpoint was very much more sensitive to the combination treatment.

As noted above, unforeseen combination effects are depending on food web interactions. For the phytoplankton, NOEC was even higher than in the single substance studies (level 2 instead of IPU1). However, this finding cannot be generalized for arbitrary pesticide combinations and ecosystematic endpoints. It is specific to the study presented here and may well be totally different if other active ingredients are used. Results in the zooplankton, especially *Eu. gracilis*, the Cyclopoids, and the species richness, indicated that the impact of the combination is stronger. In order to protect the environment from undesired side-effects of pesticide use, the most sensitive parameters has to be taken into account for risk assessment. With respect to the NOEC, the combination of IPU and CYP must therefore be considered as at least equally harmful to the investigated aquatic model ecosystem as CYP alone. The over-all NOEC for the CYP and the combined study is equal or smaller than the first pesticide level investigated. Particularly effects on the Copepods (*Eu. gracilis* and the Cyclopoids) were more pronounced in the combination, though (and started already in the first treatment level). From the results of this thesis it cannot be excluded that the NOEC of the combination might be lower than for CYP alone. Additional research using lower pesticide concentrations is needed to answer this question.

With respect to the over-all NOEC of IPU, the combination is more harmful (NOEC IPU1 with 4 µg/L IPU in the herbicide study) to the model ecosystem.

To the knowledge of the author, only three other studies in outdoor mesocosm were conducted with pesticide combinations (HINDELANG 1993, FAIRCHILD *et al.* 1994, and WENDT-RASCH 2003).

In the study of HINDELANG 1993, the direct toxic effects of carbofuran (insecticide) were more pronounced in combination with atrazine (herbizide), because the herbicide lowered the pH of the test system. Consequently, carbofuran was degraded more slowly and could exert stronger impact on Cladocerans and Copepods. In this study, the Rotifers were able to gain in abundance due to competition reasons (i.e. an secondary effect, controlled top-down, facilitated from bottom-up).

FAIRCHILD *et al.* 1994 used a combination of atrazine and esfenvalerate (insecticide). Crustacean zooplankton was reduced by the insecticide treatment alone. They stated that their combination had no different insecticide impact on the zooplankton. Reasons were the functional redundancy of the macrophytes (same standing crop, different species composition) used in their test system and the quick dissipation rate of esfenvalerate. However, they did not investigate food web interactions. Sampling was restricted to two weeks after treatment. Keeping the generation time of the zooplankton in mind (1-2 weeks for Rotifers, 2-3 months for Cladocera, and 1 year for the Copepods), it is quite obvious that complex food web

interaction like those presented in the presented study cannot be observed with such a short sampling period. However, their findings corroborate the fact that the direct toxic effects of a pesticide are comparable for susceptible taxa shortly after a combined treatment.

WENDT-RASCH *et al.* 2003 used the herbicide metsulfuron methyl and the insecticide cypermethrin. Again, sampling was limited to two weeks after treatment. Although this is not advisable (see above), they noted secondary effects (of the herbicide) on the periphyton: It increased due to nutrients leaking from the macrophytes. The phytoplankton was unaffected in their study. The zooplankton community was constituated mainly of Rotifers that were insensitive to the insecticide at the concentrations used. Consequently, no combination effects, neither direct or indirect, were observed in the zooplankton. Nevertheless, they stated: "However, the possibility of combined effects of herbicides and insecticides needs further investigation in aquatic ecosystems with different structure, in particular, in systems where crustacean zooplankton constituate an important component and secondary effects can be expected to occur." With its prolonged sampling period, the presented study has proven them right.

With further thorough research on the topic of ecosystematic reactions to combined pesticide treatment, hopefully, complex interactions might be deduced from single substance study data in the future. Therefore food web interactions must be well-known and the data provided by the researchers must not be limited to statistically significant deviations. The system reaction as a whole must be characterized to enable an interpretation of such combination effects. Special effort should be taken in determining the intrabiocoenotic control factors in the system. The results of the presented study indicate that possible combination effects might be deduced by looking at shifts in the top-down control. Whether the influence of the top-down factors is superior to the bottom-up ones in all cases of a combined pesticide action needs to be clarified in further studies. In any case, good data on the environmental fate of the substances is needed.

Predicting combination toxicity with the BLISS independence model was possible for the direct and the simpler indirect effects on biota and abiotic parameters. Further research in multi-species systems (and) in outdoor experiments is needed to prove this result, especially with fish integrated in the system and for substances with a more similar mode of action. In comprehensive studies, both wide-spread prediction models (CA *and* IA) should be tested and evaluated for risk assessment implications. In any case, the one model working is a promising outcome of this study but it can only be a starting point for more research.

8 Abstract

In the presented thesis direct and indirect (so-called secondary or systematic) reactions of plankton organisms to an insecticide and a herbicide applied jointly were investigated. The insecticide used was α-Cypermethrin (CYP), a pyrethroid, and the herbicide Isoproturon (IPU), a phenylurea. Both were chosen because they act independently from each other on a molecular basis (neurotoxin and photosystem II blocker). Addressing the toxic effects to either pesticides was enabled by this approach.

The investigation consisted of three parts: Both pesticides were applied separately in 2000 in a mesocosm experiment, and combinations of them were used in another mesocosm in 2001. Monitoring of effects in all outdoor experiments was continued for two vegetation periods. Additionally, single species tests and biomonitoring experiments were performed with zooplankton organisms in order to get a better picture of the direct (toxic) effects.

The key objectives were: examining the differences in the impact of the three pesticide treatment regimes, ascertaining how direct and secondary treatment effects of the three treatment scenarios affected the test systems ecosystematically, and finding possibilities to deduce (qualitatively) or predict (quantitatively) combination effects of the toxicant mixture.

Therefore, phyto- and zooplankton development was examined, functional parameters (water chemistry and physical properties) as well as pesticide residues were measured, and all the data were entered in thorough statistical evaluation tools. Recovery time for influenced endpoints were determined and NOEC and/or NEC data was derived using either Williams' tests or inverse regression. Functional and taxa data were entered in multivariate PRC analyses, significance levels were determined using Monte Carlo simulations and the $NOEC_{community}$ for each sampling date was derived by applying Williams' tests to the outcome of the multivariate analyses. In order to estimate the influence of environmental factors (including the pesticide residues and the sampling date as factors), CCA analysis was performed on plankton data.

The possibilities of predicting the influence of the combined treatment with the BLISS independence model (independent action) were tested.

Results of the insecticide study indicated that the sensitivity towards the active ingredient rose with the position in the food chain. The most sensitive organism was *Chaoborus crystallinus* with a NOEC of <0.015 µg/L CYP (i.e. determining the overall NOEC for this part of the thesis). This was the top predator in the system. - Recovery could be demonstrated for all endpoints. Functional parameters were not influenced. Secondary effects could therefore easily be deduced by tracking effects from top-down. The system reaction, depending on the amount of CYP used, could be derived if the sensitivity (including sub-lethal effects) of the most important taxa on each trophic level and their interaction was known. A graphical interpretation is presented.

In the herbicide study, functional parameters were affected. A distinct DO-pH-alkalinity-conductivity syndrome was observed. The system reaction had to be divided into two groups depending on whether macrophytes were influenced or not. Secondary effects were triggered from bottom-up. Oscillating patterns that were related to the grazing pressure and the amount of structural change by the effect on the macrophytes in the system were observed. Secondary

effects totally outweighed the direct toxic ones in the plankton. The overall NOEC was 4 µg/L IPU. Recovery was often linked to the re-growth of the macrophytes. Again, the system reaction is presented graphically.

In the combination, three principles for the system reaction to the pesticides used could be derived:

1. Sensitive taxa to either active ingredient showed no difference in their reaction to the independently acting pesticides unless they were integrated in very tight food web interactions.
2. The reaction of moderately susceptible taxa was altered via food web interactions. Top-down control was more important but effects only came into being if they were facilitated by a bottom-up process. Insignificant effects with a single active ingredient were sufficient for boosts in the effects of the combination treatment. Species richness in the zooplankton was affected more severely in the joint treatment and can therefore be subsumed under this group of effects. This is a strong argument against the safety of the concept of the NOEC, because in natural waters combinations of pesticides are regularly found. Protecting the environment against undesired side-effects of plant production products may not be possible with the risk assessment procedures used today. However, the overall NOEC for the combination was lower only with respect to the IPU study.
3. Taxa that were not influenced by either pesticide showed reactions in the combination study. These were of secondary nature, of course. Again, they were more intensely controlled from top-down.

The overall reaction of the parts of the test system is presented graphically. Depending on the DT_{50} of the pesticides, direct toxic effects were more important shortly after the treatment, later one secondary interaction took over. Recovery was linked to the development in macrophytes, again, for several effects.

BLISS independence was able to predict direct and simple secondary effects in taxa data and water quality parameters. This is a promising approach for risk assessment procedures. Further research on mixture toxicity (in surrogate environments) is urgently needed.

9 Zusammenfassung

In der hier vorgestellten Studie wurden direkte und indirekte (sogenannte sekundäre oder systemische) Reaktionen von Planktonorganismen auf eine kombinierte Insektizid-Herbizid-Belastung untersucht. Es wurde das Insektizid α-Cypermethrin (CYP), ein Pyrethroid, und das Herbizid Isoproturon (IPU) verwendet. Diese Wirkstoffe wurden ausgewählt, weil sie auf molekularer Ebene unabhängig voneinander wirken (Nervengift und Photosystem-II-Blocker). Daher können toxische Effekte gut auf das eine oder andere Mittel zurückgeführt werden.

Die Untersuchung setzte sich aus drei Teilen zusammen: Beide Pestizide wurden einzeln in einem Mesokosmos-Experiment im Jahr 2000 appliziert und Kombinationen davon in einer weiteren Mesokosmen-Studie im Jahr 2001. Der Beobachtungszeitraum für alle Experimente betrug zwei Vegetationsperioden. Zusätzlich wurden "single-species"-Tests und ein "Biomonitoring" durchgeführt, um die toxischen Effekte besser einschätzen zu können.

Forschungsziel war die Untersuchung von Unterschieden in der Auswirkung der drei verschiedenen Belastungsszenarien. Daraus sollten sich Hinweise darauf ergeben, wie sich direkte und sekundäre Effekte der verschiedenen Behandlungsszenarien ökosystemar auswirken können. Darauf aufbauend sollten Möglichkeiten erörtert werden, wie man die Kombinationseffekte der verwendeten Giftstoffmischungen entweder qualitativ ableiten oder quantitativ vorhersagen kann.

Funktionelle Parameter (physikochemische Eigenschaften des Wassers) und die Entwicklung von Zoo- und Phytoplankton wurden untersucht. Rückstandanalytiken auf die Wirkstoffe wurden durchgeführt. Alle Daten wurden einer gründlichen statistischen Auswertung unterzogen. Die "recovery" betroffener Endpunkte wurde untersucht. Die NOEC und/oder NEC wurden per Williams-Test beziehungsweise durch inverse Regression bestimmt. Physikochemische Messwerte und Taxadaten wurden der multivariaten Analysemethode „PRC" unterzogen. Daraus kann eine „$NOEC_{community}$" ermittelt werden, indem man die Ergebnisse der multivariaten Statistik weiteren Williams-Tests unterzieht. Um den Einfluss von Umweltvariablen (einschließlich der Pestizidkonzentration und des Probenahmetages) für die Entwicklung im Plankton abzuschätzen, wurde eine CCA Analyse berechnet.

Die Ergebnisse der Insektizidstudie zeigten, dass die Empfindlichkeit gegenüber dem Wirkstoff mit der Stellung in der Nahrungskette zunahm. Der sensibelste Organismus war *Chaoborus crystallinus* mit einer NOEC von weniger als 0.015 µg/L CYP. Damit bestimmt er auch die Gesamt-NOEC dieses Studienteils. Er stellt den wichtigsten Räuber im Testsystem dar. – "Recovery" konnte für alle Endpunkte gezeigt werden. Funktionelle Parameter wurden nicht beeinflusst. Sekundäre Effekte konnten daraus abgeleitet werden, dass man die Änderungen in der top-down-Kontrolle im Nahrungsnetz nach unten durchspielte. Abhängig von der eingesetzten Menge an CYP konnte, bei bekannter Empfindlichkeit der wichtigsten Taxa jeder trophischen Ebene gegenüber dem Wirkstoff (subletale Effekte mit eingeschlossen), die ökosystemare Reaktion abgeleitet werden. Einzige Voraussetzung war, dass die Interaktionen zwischen den Taxa gut bekannt war. Die Art der Interpretation wird grafisch vorgestellt.

In der Herbizidstudie wurden Änderungen der funktionellen Parameter festgestellt. So wurde ein deutliches "DO-pH-alkalinity-conductivity"-Syndrom beobachtet. Die Reaktion auf ökosystemarer Ebene war abhängig davon, ob die Makrophyten mit betroffen waren oder nicht. Sekundäre Effekte wurden von "bottom-up" kontrolliert. Oftmals wurde eine Reaktion beobachtet, die im Zeitverlauf stark oszillierte. Die Amplitude war abhängig vom Fraßdruck, der auf dem jeweiligen Taxon lag und von der Stärke des Wirkstoff-Effekts auf die Makrophyten und damit auf die strukturelle Integrität des Testsystems. Im Plankton überwogen die Sekundäreffekte die direkt toxischen Auswirkungen bei weitem. Die Gesamt-NOEC lag bei 4 µg/L IPU. Wiederum wurde versucht, die Systemreaktion grafisch darzustellen. Die "recovery" ist bei vielen Endpunkten an ein Nachwachsen der Makrophyten gekoppelt.

Aus der Kombinationsstudie konnten drei Prinzipien für die kombinierten Effekte der verwendeten Pestizide abgeleitet werden:

1. Die auf einen der beiden Wirkstoffe empfindliche Taxa reagierten ebenso wie bei einer Einzelbelastung, da die toxische Wirkung auf molekularer Ebene ja völlig getrennt ansetzt. Änderungen ergaben sich nur dann, wenn solch ein Taxon sehr eng ins Nahrungsnetz eingebunden war.
2. Mäßig sensible Organismen reagierten auf Grund von Wechselwirkungen über das Nahrungsnetz anders als bei einer Einzelbelastung. Die "top-down"-Kontrolle war dabei wichtiger als die "bottom-up". Manche Effekte traten allerdings nur dann auf, wenn sie durch eine Veränderung von "bottom-up" unterstützt wurden. Effekte, die bei einer Belastung mit nur einem Wirkstoff lediglich zu nicht signifikanten Auslenkungen führten, konnten in der Kombination mit einem weiteren Stressor dazu führen, dass sich Effekte potenzierten. Die Effekte in Bezug auf den Artenreichtum im Zooplankton waren in der Kombinationsstudie größer als bei den Einzelapplikationen und konnten deshalb zu dieser Gruppe gerechnet werden. Vor allem letzteres Ergebnis stellt ein ernstes Problem für die Risikoabschätzung eines Wirkstoffs über eine NOEC-Betrachtung dar. In natürlichen Gewässern finden sich regelmäßig Kombinationen verschiedenster Xenobiotika. Die Umwelt vor unerwünschten Nebenwirkungen von Pflanzenschutzmitteln zu bewahren, ist daher mit den derzeitigen Verfahren zur Risikobewertung vielleicht nicht gut möglich. Es muss jedoch darauf hingewiesen werden, dass die Gesamt-NOEC der Kombinationsstudie nur gegenüber der einzelnen Herbizidbelastung nach unten abwich.
3. Die dritte Schlussfolgerung ergab sich für Taxa, die in keiner der beiden Einzelsubstanz-Studien eine Reaktion auf die Belastung zeigten, allerdings von der Kombination betroffen wurden. Solche Effekt waren logischerweise sekundärer Natur und unterlagen wiederum stärker der "top-down"-Kontrolle. –

Die Systemreaktion wurde auch in diesem Falle grafisch dargestellt. In Abhängigkeit der Halbwertszeit der Wirkstoffe überwiegen direkt nach der Belastung die toxischen und dann die sekundären Effekte. "Recovery" hängt für einige Endpunkte wiederum von der Entwicklung der Makrophyten ab.

Das Rechenmodell der "BLISS independence" konnte toxische und einfache sekundäre Wirkungen auf Taxa und physikochemische Parameter recht treffend vorhersagen. Dies stellt ein vielversprechendes Ergebnis für die Risikoabschätzung von Umweltchemikalien dar.

Weitere Forschung im Bereich der Mischungstoxizität ist allerdings dringend erforderlich, insbesondere in ökosytemaren Studien.

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