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Consequences for derivation of environmental quality standards

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Preface

This PhD thesis was carried out at the Department of Environmental Engineering, Technical University of Denmark (DTU), between January 2007 and April 2013 under the supervision of associate professor K. Ole Kusk and professor Anders Baun. The project was co-supervised by Stefania Loutseti from IE DuPont de Nemours. The project was funded by DTU, The research school RECETO and IE DuPont de Nemours.

In the thesis the following three scientific articles are included:

Paper I: **Rosenkrantz, R.T.**, Cedergreen, N., Baun, A., Kusk, K.O. 2013a. Influence of pH, light cycle, and temperature on ecotoxicity of four sulfonylurea herbicides towards *Lemna gibba*. *Ecotoxicology*, 22 (1), 33-41.

Paper II: **Rosenkrantz, R.T.**, Baun, A., Kusk, K.O. 2013b. Growth inhibition and recovery of *Lemna gibba* after pulse exposure to sulfonylurea herbicides. *Ecotoxicol. Environ. Saf.*, 89, 89-94.

Paper III: **Rosenkrantz, R.T.**, Cupi, D., Baun, A., Kusk, K.O. 2013c. A two-hour ^{14}C-bicarbonate assimilation toxicity test using cultured algae and natural phytoplankton communities. Submitted to *Chemosphere*

The author has furthermore contributed to the following scientific papers (one of them under her maiden name Rikke Tjørnhøj) which are not included in this thesis:

Rosenkrantz, R.T, Pollino, C.A., Nugegoda, D., Baun, A. (2008). Toxicity of water and sediment from stormwater retarding basins (Melbourne, Australia) to *Hydra oligactis*. *Environ. Pollution*, 156 (3), 922-927.

Andersen, T.H., Wollenberger, L., Slothuus, T., **Tjørnhøj, R.**, Baun, A. (2006). Acute and chronic effects of pulse exposure of *Daphnia magna* to dimethoate and pirimicarb. *Environ. Toxicol. Chem.*, 25 (5) 1187-1195.

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from: DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, reception@env.dtu.dk.

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Also, a special thanks to Cecilie, Charlotte, and Susanne for friendship and encouragement, and to Signe and Susanne for all the help and nice talks in the laboratory. Moreover, I thank my co-authors Nina Cedergreen and Denisa Cupi.

Finally, I would like to thank my husband, my children, and the rest of my family for the loving support and never-ending faith in me.

Summary

Chemicals are used extensively and are part of many aspects of human life and society. They are useful and necessary for many purposes, but may also cause adverse effects on natural organisms if they are released to the environment during and after use. Therefore, it is necessary to perform a risk assessment to predict and prevent adverse effects. In Europe, chemical registration and safety is regulated by the European Commission by the implementation of different directives, such as REACH (Registration, Evaluation, Authorization, and restriction of Chemicals, EC 1907/2006), the Biocide Directive (98/8/EC) or the Plant Protection Products Directive (EC 1107/2009). In addition to such risk assessment directives, the Water Framework Directive (2000/60/EC) has been adopted with the objective to protect and improve the quality of European waters and aquatic habitats. This requires, among other things, an assessment of compliance with environmental quality standards (EQS) for xenobiotic chemicals and metals. The basis for the derivation of these EQS is the focus of this thesis.

An EQS is the concentration of a chemical, below which no adverse effect of the ecosystem is expected to occur. It will often be calculated from results of ecotoxicological tests performed according to internationally approved guidelines, such as from the Organisation for Economic Co-operation and Development (OECD) or International Standardization Organisation (ISO). Such guidelines were originally developed to enable classification and hazard ranking of chemicals, and therefore their focus is to measure the toxicity of an artificially maintained continuous exposure under test conditions that rarely reflect natural conditions. This may be in contrast to the aim of establishing EQS, i.e. to protect the natural ecosystem from chemical stress. In light of this possible contradiction, the aim of this thesis was to investigate whether EQS derived on the basis of guideline tests will be sufficiently protective of the environment. This was done by exploring the influence of a number of test conditions, such as temperature, light, pH and exposure duration on the toxicity recorded in tests using four sulfonylurea herbicides (SUs) and the aquatic macrophyte *Lemna gibba* as study objects.

The study showed that changing the physical and chemical test conditions influenced the toxicity of sulfonylurea herbicides towards *L. gibba*. Lowering the temperature from 24 to 15°C caused a two-fold reduction in the toxicity of flupyrsulfuron-methyl, while no statistically significant changes was seen for metsulfuron-methyl, rimsulfuron, or thifensulfuron-methyl. Likewise, the introduction of a 12:12-hours light:dark cycle resulted in a two-fold reduction of

the toxicity of thifensulfuron-methyl. Again, no clear trend or statistically significant change was observed for the other three test SUs. Finally, the toxicity of the four SUs was tested at three different pH levels (6, 7.5 and 9). Here, it was observed that the difference in toxicity between tests at pH 6 and 9 was in the range of a factor 2-10 for the four SUs, with the tests at pH 6 being most toxic. Hence, the conclusion of this study was that by performing only guideline tests there may be a risk of underestimating the toxicity of a compound. It was recommended to carefully evaluate the physico-chemical properties of the compound prior to testing and to design the test to include the influence of these on the toxicity observed.

The effect of a 24-hour pulse of the four SUs was evaluated in another part of the study. It was shown that these short-term high-concentration exposures resulted in EC50 values that were 2-6 times higher than those obtained in guideline tests. Within the WFD, a short-term water quality standard (MAC-EQS) is derived, that should protect against effects from pulse discharges, however when this is compared to the result of the pulse tests from this study, it appears that the WFD approach is under-protective. Hence, a revision of the WFD approach should be considered because protection from pulsed discharges should not be based on the results of tests with a continuous exposure, even if they are short-term tests. Instead, the derivation of a MAC-EQS should be based on pulse tests and possibly also modelling and it should consider the recovery of the organism

The overall conclusion of this thesis is that under the current approach for derivation of EQS, there will be cases where basing the value on results from guideline tests alone will not be sufficiently protective of the environment. However, there are also be cases where the choice of test design would possibly overestimate the toxicity, and consequently the resulting EQS would be too strict. Bringing more environmental realism into the testing by designing tests according to the physico-chemical properties, and taking the use pattern of the compound into consideration, would probably result in a better estimation of the effects and thereby the EQS.

Dansk sammenfatning

Kemikalier anvendes overalt og indgår dermed i mange af dagliglivets aspekter. De er nyttige og uundværlige til mange formål, men kan også forårsage skadelige effekter på organismer i miljøet, hvis de frigives under eller efter brug. Derfor er det nødvendigt at risikovurdere for at forudsige og forhindre eventuelle skadelige effekter af kemikalier. I Europa er registreringen og sikkerheden af kemikalier reguleret af den europæiske kommission ved implementeringen af forskellige direktiver så som REACH (registrering, evaluering, autorisation og restriktion af kemikalier, EC 1907/2006), biociddirektivet (98/8/EC) og plantebeskyttelses-direktivet (EC 1107/2009). Ud over disse risikovurderingsdirektiver er der vandrammedirektivet (2000/60/EC), som blev vedtaget med det formål at beskytte og forbedre kvaliteten af europæiske vandområder og –habitater. Dette kræver blandt andet en vurdering af overensstemmelsen med miljø-kvalitetskrav (EQS) for miljøfremmede kemikalier og metaller. Fokus for denne ph.d. afhandling er de tests som danner basis for fastsættelse af miljøkvalitetskrav.

Et miljøkvalitetskrav er koncentrationen af et kemikalie, hvorunder der ikke forventes at opstå skadelige effekter på økosystemet. Det beregnes ofte på grundlag af resultater fra økotoksikologiske tests udført i henhold til internationalt godkendte vejledninger, såsom fra Organisationen for økonomisk samarbejde og udvikling (OECD) eller den international organisation for standardisering (ISO). Oprindeligt blev disse vejledninger udviklet for at gøre det muligt at klassificere og rangordne kemikalier med hensyn til fare. De er derfor målrettet målingen af toksicitet ved en kunstigt vedligeholdt og kontinuerlig eksponering til kemikaliet under test betingelser som sjældent afspejler naturlige forhold. Dette står i kontrast til formålet med fastsættelse af EQS, dvs. beskyttelsen af naturlige økosystemer mod kemisk stress. I lyset af denne mulige selvmodsigelse er formålet med denne ph.d. afhandling at undersøge, hvorvidt EQS fastsat på baggrund af disse vejledninger er tilstrækkeligt beskyttende for miljøet. Dette blev gjort ved at udforske indflydelsen af en række testbetingelser, såsom temperatur, lys, pH og eksponeringens varighed, på toksiciteten målt ved tests udført med fire sulfonylurea herbicider og den akvatiske makrofyt *Lemna gibba* som forsøgsobjekter.

Studiet viste at ændringen af de fysiske og kemiske testbetingelser havde indflydelse på toksiciteten af sulfonylurea herbicider på *L. gibba*. En sænkelse af temperaturen fra 24 til 15 °C gav en halvering af toksiciteten af flupysulfuron-methyl, mens der ikke blev set statistisk signifikante ændringer for metsulfuron-methyl, rimsulfuron eller thifensulfuron-methyl. Ligeledes resulterede

indførelsen af en 12:12 timers lys:mørke cyklus i en halvering af toxiciteten af thifensulfuron-methyl. Igen blev der ikke observeret nogen tydelig eller statistisk signifikant ændring for de andre tre test sulfonylurea herbicider. Endelig blev toksiciteten af de fire sulfonylurea herbicider testet ved tre forskellige pH niveauer (6, 7.5 og 9). Her blev det observeret, at ændringen i toksicitet mellem testene ved pH 6 og 9 var inden for en faktor 2-10 for de fire sulfonylurea herbicider, hvor testene ved pH 6 var mest toksiske. Derfor er konklusionen af dette studie at ved udførelsen af tests som følger OECD vejledningen, kan der være en risiko for at underestimere toksiciteten af et kemikalie. Det anbefales derfor, at evaluere kemikaliets fysisk-kemiske egenskaber grundigt forud for testning, og at testen designes så indflydelsen af disse egenskaber på toksiciteten undersøges.

I en anden del af studiet blevet effekten af en 24 timers puls af hver af de fire sulfonylurea herbicider undersøgt. Det blev vist, at en kort puls med en høj koncentration resulterede i effektværdier (EC50), som var mellem to og seks gange højere end de, der blev fundet i tests udført efter OECD vejledningen. Sammenlignes denne forskel med det kort-tids-vandkvalitetskriterier (MAC-EQS), som fastsættes under Vandrammedirektivet, ser det ud til, at Vandrammedirektivet vil være underbeskyttende. Derfor anbefales det, at retningslinjerne for fastsættelse af MAC-EQS laves om, så beskyttelse mod pulsudledninger ikke er baseret på tests med kontinuert eksponering, også selvom om det er korttidstests. Fastsættelsen burde i stedet være baseret på puls tests, som den her viste, og eventuelt også modellering. Derudover burde det være muligt at tage hensyn til, om den eksponerede organiske restituerer sig, da det ofte er set efter en pulseksponering.

Den overordnede konklusion på dette projekt er, at ved den nuværende tilgang til fastsættelse af EQS, vil der være tilfælde, hvor det at basere værdien på resultater fra forsøg udført efter OECD vejledninger, ikke vil være tilstrækkeligt beskyttende for miljøet. At tilføre undersøgelserne mere miljømæssig realisme ved at designe forsøgene i henhold til de fysisk-kemiske værdier, og tage brugsmønstret af stoffet i betragtning, ville efter al sandsynlighed resultere i et bedre estimat af effekterne og dermed fastlæggelse af EQS.

List of abbreviations

AA-EQS	Annual Average Environmental Quality standard
AF	Assessment Factor
ALS	Acetolactate Synthase
EC	European Commission
ECHA	European Chemicals Agency
EFSA	European Food Safety Agency
EQS	Environmental Quality Standard
HC5	Hazardous concentration, 5th-percentile of the SSD
L(E)C10	Concentration causing 10% Effect or Lethality
L(E)C50	Concentration causing 50% Effect or Lethality
MAC-EQS	Maximum Allowed Concentration Environmental Quality Standard
MAD	Mutual Acceptance of Data
NOEC	No Observed Effect Concentration
OECD	Organisation for Economic Co-operation and Development
PAH	Poly Aromatic Hydrocarbon
PCP	Pentachlorophenol
PHS	Priority Hazardous Substance
pKa	Acid dissociation constant
PNEC	Predicted No Effect Concentration
PPP	Plant Protection Product
PS	Priority Substance
RBD	River Basin District
REACH	Registration, Evaluation, Authorization, and restriction of Chemicals
SSD	Species Sensitivity Distribution
SU	Sulfonylurea
TGD	Technical Guidance Document
TWA	Time-Weighted Average
WFD	Water Framework Directive

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Readers guide

Chapter 1 - Introduction

Chapter 1 explains the background and reasons for choosing this area of research as well as stating the aims of the thesis.

Chapter 2 – The Water Framework Directive

This chapter describes the Water Framework Directive (European Commision, 2000a) and in particular the principles for derivation of environmental quality standards given in the Technical Guidance Document (European Commission, 2011a).

Chapter 3 – The influence of test conditions

This chapter begins with a description of the OECD Standard test programme and continues with an overview of some important test conditions and what influence changes of these conditions may have on the ecotoxicity of chemicals.

Chapter 4 – The influence of exposure duration

Chapter 4 gives an overview of the research within effects of pulse exposures in ecotoxicity tests.

Chapter 5 - Discussion

Findings of Chapter 3 and 4 are put into the context of the Water Framework Directive. It is discussed whether the use of guideline tests is appropriate to give a reliable protection of the ecosystem and if pulse exposures are addressed adequately within the current approach.

Chapter 6 - Conclusion

Here the conclusions from the research are given and recommendations for the use of guideline tests for derivation of environmental quality standards are highlighted.

1 Introduction

1.1 Background

Chemicals are an integral part of human life today and they contribute to our well-being in a vast number of ways. Pharmaceuticals help to cure both humans and animal from disease, dyes are used to colour fabric, pesticides are used for crop protection and increasing yield, etc. During and after use, chemical emissions to the environment are unavoidable and hence, there is a risk of adverse effects on natural organisms. It is therefore necessary to perform a risk assessment of a chemical to predict and prevent possible adverse effects on the ecosystem.

In Europe, chemical registration and safety is regulated by the European Commission (EC) by implementation of directives into the national legislation of the member states. Chemicals are grouped according to their use and accordingly, there are several directives dealing with chemical risk assessment. Currently, the following chemical groups require an environmental risk assessment: Industrial chemicals (Directive EC 1907/2006), Plant Protection Products (PPP) (EC 1107/2009), biocides (98/8/EC), veterinary pharmaceuticals (2001/82/EC), and pharmaceuticals for human use (2001/83/EC). Moreover, the Water Framework Directive (WFD) (2000/60/EC) requires that Environmental Quality Standards (EQS) be set for chemicals that are likely to be discharged to the aquatic environment.

Both chemical risk assessment and EQS derivation consists of an effect part and an exposure part, which is compared in order to determine if a risk is present or an exceedance of the EQS has occurred. However, the risk assessment of chemicals under for example REACH represents a predictive assessment, while EQS derivation is a retrospective assessment, where the difference lies within the exposure part. Risk assessment is based on predicted or modelled environmental concentrations, which are calculated according to the recommended use of the chemical, while the exposure part of the EQS derivation is based on monitoring results, i.e. on the actual concentration measured in the environment. For both types the effects assessment part is similar in the respect that it is based on the results of ecotoxicological tests. This thesis will be concentrated on the effects assessment part and interpretations of the recommendations given in the Technical Guidance for derivation of EQS under the WFD (European Commision, 2011a).

The effect assessment is based on available data from toxicity tests carried out in the laboratory and/or from mesocosm or field studies. However, due to a lack of data, effect assessment will often only be based on results of toxicity tests performed according to an internationally approved guideline or standard protocol (Ågerstrand et al., 2011). Standardized test protocols and guidelines were developed with the aim of quantifying toxic effects as a function of the concentration of the tested compound, and were originally intended for classification and ranking of chemicals (Diderich, 2007). This requires, among other things, that test conditions and exposure concentrations are kept constant throughout the incubation period, and this may cause the use of this kind of tests in EQS derivation (and risk assessment) to be problematic. The test conditions (e.g. light, temperature and pH) in a laboratory test rarely reflect the natural conditions in the environment (Laskowski et al., 2010), and since the EQS is made to protect the natural ecosystem, it is important to investigate if it is appropriate to use guideline tests for EQS derivation and risk assessment. The maintenance of constant exposure conditions in guideline tests has become increasingly more difficult to achieve since chemical complexity is increasing (Backhaus et al., 2012). Many chemicals are difficult to handle because they sorb, volatilise, ionise and degrade or transform during the course of a tests, making it hard to determine the actual exposure concentration and duration. Therefore, it is also important to investigate the appropriateness of using tests that were intended for use with chemicals that are easily maintained at constant concentration, for tests with substances that disappear or transform during the test.

1.2 Aims

The overall research question of this thesis is: Will the resulting environmental quality standards be sufficiently protective if the above concerns regarding test conditions and exposure duration are ignored?

The thesis aims to address this question by:

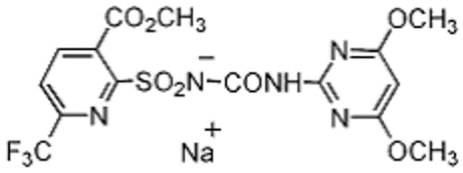
1. Investigating and describing the influence of physical and chemical test conditions on ecotoxicological effects.
2. Examining the importance of the additional information gained by performing pulse exposure test compared to ordinary guideline tests.
3. Evaluating if and how the above findings may be applied in current procedures for derivation of environmental quality standards and the implications hereof.

1.3 Test objects

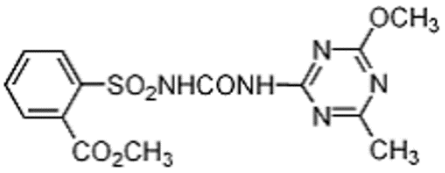
A common feature of the scientific articles included in the thesis is the use of four sulfonylurea herbicides (SUs) as model compounds: Flupyrsulfuron-methyl, metsulfuron-methyl, rimsulfuron, and thifensulfuron-methyl. These compounds were chosen because they are highly relevant to this study due to the properties mentioned below:

- Direct exposure of the environment during application.
- Among the most widely used herbicides
- High specific toxicity at very low doses
- Degradation and/or transformation behaviour during ecotoxicity testing
- Weak organic acids with pH dependent hydrolysis half-life, water solubility, and logKow (Table 1).

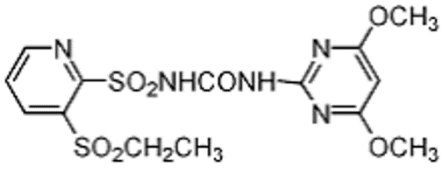
SUs are specifically acting herbicides belonging to the group of ALS (acetolactate synthase) inhibitors. The herbicides inhibit the ALS enzyme, which leads to cessation of cell division, and subsequently inhibition of the growth processes in the plant (Cobb, 1992). Animals lack the ALS enzyme and therefore SUs exhibit low toxicity in traditional ecotoxicity tests with fish and crustaceans, but have shown to be very toxic to aquatic plants. Especially *Lemna* species (Duckweed) have been found to be very sensitive to SUs (Cedergreen et al., 2004; Cedergreen et al., 2004; Cedergreen and Streibig, 2005) and are therefore likely to be one of the defining organisms for the effect assessment of SUs (see Table 2 for effect values). Thus, *Lemna gibba* was chosen as the primary tests organism.



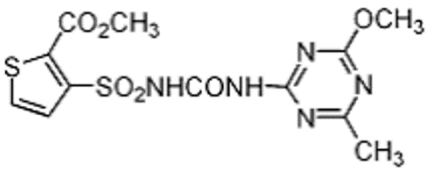
Flupyrsulfuron-methyl



Metsulfuron-methyl



Rimsulfuron



Thifensulfuron-methyl

Figure 1. Chemical structures for the four sulfonylurea herbicides used as model compounds.

Table 1. Physico-chemical properties for the four sulfonylurea herbicides (European Commission, 2001b; European Commission, 2001a; European Commission, 2000b; EFSA, 2005).

		Flupyrsulfuron-methyl	**Metsulfuron-methyl**	**Rimsulfuron**	**Thifensulfuron-methyl**
CAS No.		144740-54-5	74223-64-6	122931-48-0	79277-27-3
Molecular mass		487.4	381,4	431.45	387.4
pKa		4.94	3.75	4.0	4.0
Vapor pressure (pa, T= 20°C)		$< 1 \cdot 10^{-9}$	$1.1 \cdot 10^{-10}$	$8.9 \cdot 10^{-7}$	$7.5 \cdot 10^{-9}$
Henry's law constant ($Pa \cdot m^3 \cdot mol^{-1}$)	pH5	$< 10^{-8}$	$2.3 \cdot 10^{-10}$	$4.6 \cdot 10^{-6}$	$1.3 \cdot 10^{-12}$ (unknown pH)
	pH6	$< 10^{-9}$	n.a.	n.a.	
	pH7	n.a.	$4.5 \cdot 10^{-11}$	$8.3 \cdot 10^{-8}$	
	pH 9	n.a.	n.a.	$1.1 \cdot 10^{-7}$	
Water solubility (g/L, T=25°C)	pH 5	0.06 (20°C)	0.548	0.135	0.223
	pH 6	0.61 (20°C)	n.a.	n.a.	n.a
	pH 7	Unstable (20°C)	2.79	7.3	2.24
	pH 9	n.a.	213	5.56	8.83
$\log K_{OW}$ (25°C)	pH 5	0.96*	n.a.	0.288	1.06
	pH 6	0.06	n.a.	n.a.	n.a.
	pH 7	n.a.	-1.7	-1.46	- 1.7
	pH 9	n.a.	n.a.	n.a.	- 2.10
Hydrolytic stability, DT50 (days)	pH 5	44	22	4.7	5.5
	pH 7	12	>30	7.3	190
	pH 9	0.42	No degradation	0.18	92
Photostability in water (DT50 days)	pH 5	470	No degradation	1.1	4.1
	pH 7	33	No degradation	11.7	5.2
	pH 9	5.4	No degradation	0.46	4.0

n.a.: Data not available

*stated as: 9.17 (Pow = 0.96) in the reference (European Commission, 2001a) but this was evaluated to be erroneous.

Table 2. Ecotoxicity values (mg/L) for the four sulfonylurea herbicides towards fish, crustacean, algal, and macrophyte species.

		Flupyrsulfuron-methyl		**Metsulfuron-methyl**		**Rimsulfuron**		**Thifensulfuron-methyl**	
		Value	Ref.	Value	Ref.	Value	Ref.	Value	Ref.
Fish		mg/L		mg/L		mg/L		mg/L	
Oncohynchus mykiss	$LC50_{96h}$	470	[1]	>150	[2]	>390	[3]	>100	[4]
	$NOEC_{28d,\ growth}$	130	[1]	68	[2]	125	[3]	250	[4]
Crustaceans		mg/L		mg/L		mg/L		mg/L	
Daphnia magna	$EC50_{48h,\ immobilisation}$	721	[1]	>150	[2]	>360	[3]	470	[4]
	$NOEC_{21d,\ growth}$	16	[1]	150	[2]	1	[3]	100	[4]
Algae		µg/L		µg/L		µg/L		µg/L	
*Pseudokirchneriella subcapitata**	$EC50_{72h,\ growth}$	3.7	[1]	45	[2]	1200	[3]	15.9	[4]
	$EC50_{48h,\ growth}$	N.A.		677	[6]	N.A.		N.A.	
	$EC10_{48h,\ growth}$	N.A.		292	[6]	N.A.		N.A.	
Anabeana flos-aquae	$EC50_{72h,\ biomass}$	99	[1]	N.A.		N.A.		N.A.	
Macrophyte		µg/L		µg/L		µg/L		µg/L	
*Lemna gibba***	$EC50_{14d,\ biomass}$	2.5	[1]	0.36	[2]	4.6	[3]	1.3	[4]
	$EC50_{7d,\ pH\ 6,\ fronds}$	0.19	[5]	0.64	[5]	0.43	[5]	0.96	[5]
	$EC50_{7\ d,\ pH\ 7.5,\ fronds}$	0.95	[5]	0.37	[5]	1.1	[5]	2.0	[5]
	$EC50_{7\ d,\ pH\ 9,\ fronds}$	2.1	[5]	1.4	[5]	1.2	[5]	3.4	[5]
	$EC10_{7d,\ pH\ 6,\ fronds}$	0.016	[5]	0.12	[5]	0.022	[5]	0.092	[5]
	$EC10_{7\ d,\ pH\ 7.5,\ fronds}$	0.12	[5]	0.27	[5]	0.16	[5]	0.34	[5]
	$EC10_{7\ d,\ pH\ 9,\ fronds}$	0.72	[5]	0.27	[5]	0.23	[5]	1.3	[5]
*Lemna minor***	$EC50_{7\ d,\ pH\ 5,\ frond\ area}$	N.A.		0.79	[6]	N.A.		N.A.	
	$EC10_{7\ d,\ pH\ 5,\ frond\ area}$	N.A		0.37	[6]	N.A		N.A	
Myriophyllum spicatum	$EC50_{14\ d,\ plant\ length}$	N.A.		1.00	[7]	54	[7]	3.4	[7]
8 different macrophytes	$EC50_{14d,\ Specific\ leaf\ ares}$	N.A.		0.1-2.21	[8]	N.A.		N.A.	

* Formerly known as *Selenastrum capriconutum.*

*** "OECD Guideline 221: Lemna sp. Growth Inhibition Test. For this 7-d test with duckweed the same considerations can be made as for the algal test (OECD 201): the EC50 from this test is considered an acute value, the NOEC or EC10 a chronic value."* (European Commision, 2011a).

References: [1]European Commission (2001a); [2] European Commission (2000b); [3] EFSA (2005); [4] European Commission (2001b); [5] Rosenkrantz et al. (2013a); [6] Cedergreen and Streibig (2005); [7] Turgut and Fomin (2002); [8] Cedergreen et al (2004).

2 The Water Framework Directive

2.1 General introduction

The Water Framework Directive (WFD, 2000/60/EC) (European Commission, 2000a) was adopted in 2000 in the realisation that a more integrated approach was needed to protect the water bodies in the European Union (EU) and restore them to a good biological and chemicals status. Before the WFD, EU water policy was a patchwork of different regulations covering different types and groups of pollutants (urban waste water, dangerous substances, nitrate etc.) and different types of waters and their use (drinking water, bathing water, fishing waters, ground water etc.). Some of these directives were collected under the WFD, while other directives are still in place with the WFD acting as an overall reference providing a complete water policy in the EU. The main objective of the WFD is to protect water quality by cleaning polluted waters and ensuring that clean waters are kept clean. Originally, the WFD covered both surface water and ground water, but in 2006 the Groundwater Directive (2006/118/EC) concerning protection of groundwater against pollution and deterioration was adopted. Though the interaction between groundwater and surface water is a part of the WFD, assessment of groundwater quality is out of the scope of this thesis, and all references to water quality etc. will in the following be related to surface water.

One of the major changes in European water management incurred by the WFD is that water management is now made according to river basins (natural geographical and hydrological units) and not according to administrative or political boundaries. This means, that when a river basin district (RBD) crosses a national border, the authorities in the respective countries have to collaborate on the water management in that district. For each RBD a management plan has to be made, where the different surface water bodies (rivers, lakes, transitional waters, and coastal waters) are evaluated and their ecological and chemical status classified based on monitoring data.

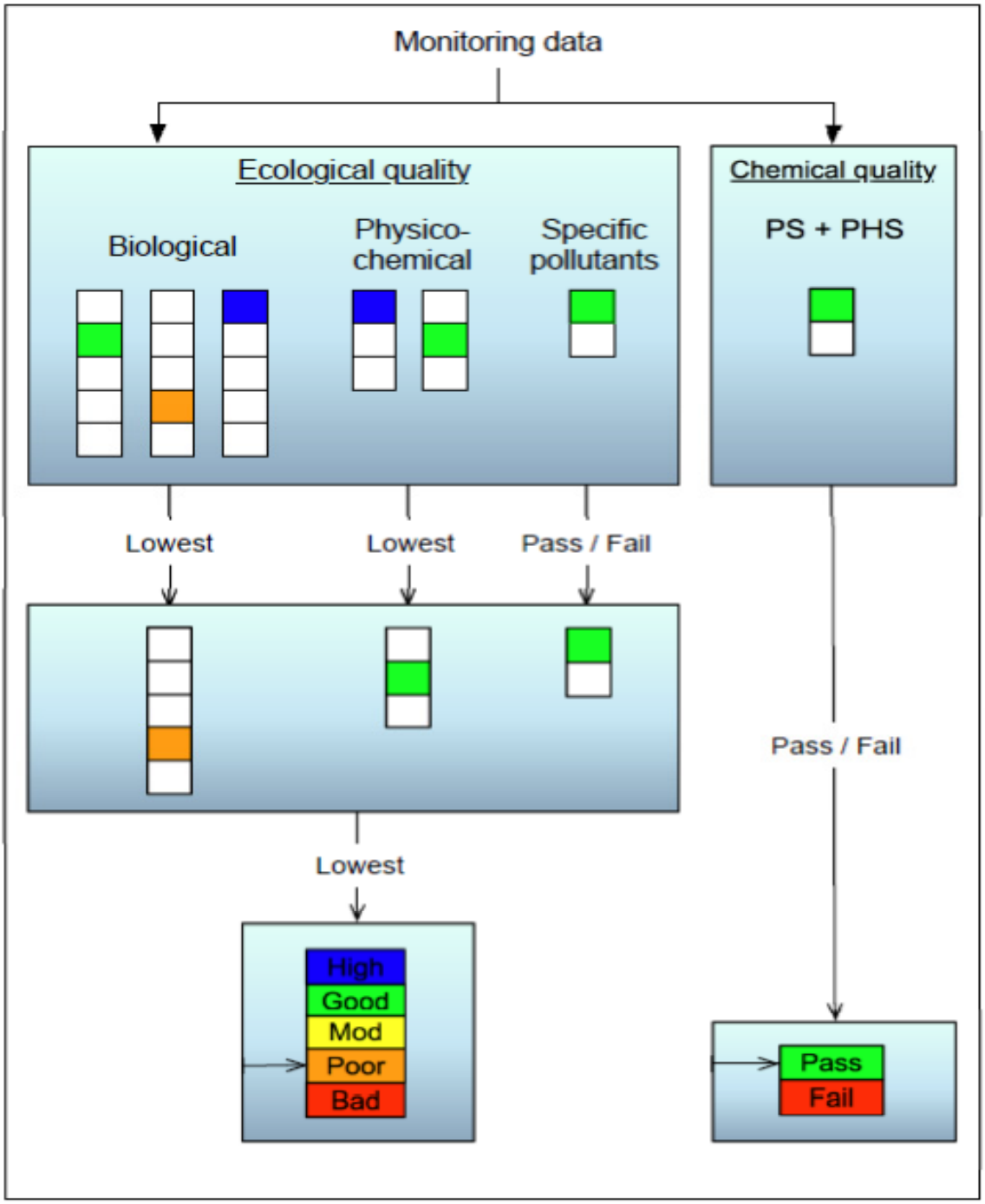


Figure 2. Classification of surface water status is comprised by ecological and chemical quality. For biological- and physico-chemical quality elements, monitoring data are compared with values from a reference waterbody, and the lowest quality score determines the classification. Measured concentrations of specific pollutants (under ecological quality) and Priority- and Priority Hazardous Substances (PS + PHS) (under chemical quality) are compared with Environmental Quality Standards (EQS). If the measured concentration is above the EQS, the waterbody fails to meet the objective of good status, and if it is below, the status is determined by the lowest scoring quality element (European Commission, 2011a).

2.2 Surface water status

Another primary principle of the WFD is that the status of a waterbody is assessed with regards to chemical and ecological quality (Figure 2), and is recorded as high, good, moderate, poor or bad. The status "High" is given to waters with largely undisturbed conditions, while the other classes deviate increasingly from undisturbed (or reference) conditions. The value for each element (obtained via monitoring) is compared to a reference state, and the waterbody is classified relative to this reference state with the overall status being determined by the lowest quality element status.

The chemical status considers only Priority Substances (PSs) and Priority Hazardous Substances (PHSs). There are currently 45 PSs of which 17 are considered PHSs. The PSs are a diverse group containing both pesticides, heavy metals, PAHs, PCBs and other chemicals and chemical groups. The measured concentrations of these compounds in a waterbody are compared with Environmental Quality Standards (EQS) and the chemical status is recorded as "pass" or "fail" (Figure 2). The chemical status is determined by the worst scoring chemical (one-out-all-out approach). EQS for PSs are determined at EU level because these substances are given priority due to their high potential for pollution regardless of region and waterbody type. The current EQS for PSs are found in the Environmental Quality Standards Directive (2008/105/EC) (European Commission, 2008), but a proposal for a new EQS Directive has been published where additional substances are included and some EQS are changed (European Commission, 2011b).

The ecological status is comprised by the status of three quality elements: biological quality elements, physico-chemical quality elements, and an assessment of specific pollutants. The biological status is assessed by the composition and abundance of aquatic flora and fauna, and of the presence of alien species.. The physico-chemical quality covers parameters such as pH, oxygen content, temperature, salinity, hydromorphological elements (e.g. water flow and physical habitat) etc., while the specific pollutants assessment (within the ecological classification) refers to the compliance with EQS for specific pollutants. These are substances that are discharged in significant quantities to a waterbody and which could adversely affect the ecology. An EQS should also be determined for specific pollutants and this value should be set by the member state in which the discharge is made. Hence, these values may be different from member state to member state due to differences in the interpretation of data and environmental differences (climate, geology etc.) between regions.

2.3 EQS derivation

As mentioned in 2.2 EQS are essential in the assessment of both chemical and ecological status. Furthermore, they are used in determination of discharge permits in order to prevent EQS exceedance within receiving waters. The method for determination of EQS, which is briefly outlined in Annex V of the WFD, is based on the principles used in the risk assessment of chemicals under REACH (ECHA, 2008). However, the hazard assessment paradigm under REACH relies on worst-case assumptions, which is legitimate because it ensures that the environment is protected, but such a conservative approach may lead to unrealistically low EQS. In order to prevent this, a Technical Guidance Document (TGD) for derivation of EQS was published by the European Commission (EC) in 2011 (European Commision, 2011a).

The TGD provide guidelines for derivation of EQS for the water column, biota and sediment, and furthermore it gives detailed advice for derivation of EQS for metals and mixtures, and for the use of non-testing methods (European Commision, 2011a). The following will only describe the guidance concerning water column EQS, since other environmental compartments are not relevant for this project. Likewise, details for metals and mixtures are not concerned.

For the water compartment, two quality standards are necessary. For protection against long-term exposure an Annual Average Quality Standard (AA-QS) is derived and for protection against short-term exposure a Maximum Acceptable Concentration (MAC-QS) is derived. The derivation of both standards is based on the results of ecotoxicological test, but while the AA-QS is preferably based on chronic toxicity data, the MAC-QS only relies on acute data.

There are three different approaches for derivation of the AA-QS, namely the deterministic approach, using the Assessment Factor (AF) method, the probabilistic method, using species sensitivity distributions (SSD), and finally using results from model ecosystems and field studies. The term “assessment factor method” is somewhat misleading, since all three approaches use assessment factors (Table 3) to account for uncertainties in data, such as inter- and intra-species variation and laboratory to field extrapolation (European Commision, 2011a). The appropriate approach to use is decided by the amount of data available, however, if enough data is available EQS should be derived using all three methods.

A distinction is made between freshwater and saltwater by the application of different AFs. This means that although data from ecotoxicity tests with freshwater species may, in many cases, be used as the basis for the saltwater EQS, the resulting EQS will generally differ from the one derived for freshwater due to higher assessment factors for marine waters. In the following, the methods are described using AFs for freshwater.

The AF method

The AF method is the method that requires the least amount of data. It uses results from ecotoxicological tests with organisms from the base set (algae and/or macrophytes, daphnia and fish), which represents three different trophic levels. Test can be short-term or long-term, and results from these are expressed as L(E)C50 values and NOEC/EC10, respectively.

Table 3. Assessment factors for derivation of Environmental Quality Standards for freshwater (European Commision, 2011a).

Available data	**Assessment Factor**
At least one short-term L(E)C50 from each of three trophic levels of the base set	1000
One long-term NOEC or EC10 (either fish or daphnia)	100
Two long-term results (NOEC or EC10) from species representing two trophic levels (fish and/or daphnia and/or algae)	50
Long-term results (NOEC or EC10) from at least three species (normally fish, daphnia and algae) representing three trophic levels	10
Species Sensitivity distribution (SSD)	5-1 (to be fully justified case by case)
Field data or model ecosystems	Reviewed on a case-by-case basis

The procedure for setting the AA-EQS is to divide the lowest effect value available by the appropriate assessment factor. If for example short-term values for all three species in the base set are available along with long-term results for daphnia and fish, the lower of the two long term values is divided with 50 and this value becomes the EQS. At first glance this method seems straight forward, but there are many exceptions and special cases to be aware of, which may complicate the derivation.

The SSD method

With the probabilistic method a SSD is constructed by log-transforming and fitting data to a distribution function (often the log-normal distribution, but others may be used). From this distribution a percentile (usually the 5th percentile,

referred to as the HC5) is used as the basis for the EQS derivation (European Commision, 2011a). The input data for the SSD should be results from long term tests (NOEC or EC10) and it should be representative of the community of interest, i.e. the resulting EQS should be protective of the whole ecosystem. Therefore, the dataset from which the SSD is constructed should contain preferably more than 15, but at least 10, test results from at least 8 taxonomic groups (European Commission, 2011a). Furthermore, for substances with a specific mode of action, two SSDs should be constructed: One covering the whole ecosystem as mentioned above, and one using the taxa expected to be particularly sensitive to the compound in question (e.g. for a herbicide (intended to kill plants), data for higher plants and algae would be used). The AF applied to the HC5 from the SSD (Table 3) is usually set to 5, but it may be lowered if evidence can remove residual uncertainty, e.g. when data quality and the number of taxonomic groups are high, and statistical uncertainty around the HC5 is low.

Use of field or mesocosm studies

Field studies and simulated ecosystem studies (micro- and mesocosms) are often used in the risk assessment of pesticides, and can be a valuable tool in the evaluation of chemical impact on ecosystems, because they present more environmentally realistic conditions than standard single-species laboratory tests. A NOEC/EC10 from a field or mesocosm study may either be used as the basis for the EQS derivation or as support in the selection of the size of the AF for a SSD, because it provides a valuable link between laboratory and field data (European Commission, 2011a). The AF applied to a NOEC from a field or mesocosm study is usually set to 5, but may be adjusted up or down depending on the number of studies and whether sensitive species are sufficiently represented (European Commission, 2011a).

MAC-EQS derivation

As mentioned above, the MAC-EQS is required to protect the ecosystem from effects from short term concentration peaks, also called pulses. A method for this derivation was developed in the REACH TGD (ECHA, 2008), and this method is directly adopted for the derivation of a MAC-EQS under the WFD. Under REACH, pulses are defined as discharges *"occurring infrequently, i.e. less than once per month and for no more than 24 hours"* (ECHA, 2012). It should be noted that the MAC-EQS cannot be lower than the AA-EQS. This would make little toxicological sense, since chronic effects usually occur at lower exposure

concentrations than acute effects. If this should however be the result of the extrapolations, the MAC-EQS is set equal to the AA-EQS.

The derivation of the MAC-EQS can be done using the same three methods as for the AA-EQS (AF method, SSD method, and field data method), but using L(E)C50 values from short term tests instead of NOEC/EC10 values from long term tests. With the AF method, the MAC-EQS is derived by applying an AF of 100 to the lowest L(E)C50 value of the base set. In special cases, such as where the mode of toxic action is known or the most sensitive species is included in the data set, the AF can be lowered to 10 (European Commision, 2011a). For the SSD method, the same approach is used as for the deriving the AA-EQS, only using short term data as input. An AF of 10 should be applied to the HC5 of the SSD, unless other lines of evidence suggest that a higher or lower would be appropriate. If mesocosm studies are available for substances where the mode of action of the compound is known or the most sensitive species is known, an AF of 1-5 is applied to the lowest L(E)C50 from that study (European Commision, 2011a).

2.4 Data evaluation

Data collection and evaluation is one of the largest tasks in EQS derivation. There are many factors that could influence the size of the EQS and it is important to know all relevant information (European Commision, 2011a).

Physico-chemical data, such as water solubility, vapour pressure, photolytic and hydrolytic stability, and acidic or basic properties, are important for the evaluation of ecotoxicological tests because they reveal how the compound behaves in the tests solution and if there is a risk that it might disappear during the test.

All ecotoxicological test results should be used (providing they are relevant and reliable), since this decreases the uncertainty of the resulting EQS. If multiple data are available for the same species and endpoint, the data points may be aggregated into a single value. However, it must be ensured that differences in toxicity is not due to different test conditions, test exposure duration, test compound form etc. (European Commision, 2011a).

When all available data has been collected it must be quality assessed with regards to both reliability and relevance. As in the REACH TGD (ECHA, 2011a), the reliability of a study is evaluated by designation of quality codes

according the scheme developed by Klimisch et al. (1997), as cited in ECHA (2011a):

1. ***Reliable without restrictions:*** *'studies or data...generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline...or in which all parameters described are closely related/comparable to a guideline method.*
2. ***Reliable with restrictions:*** *'studies or data...(mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable.'*
3. ***Not reliable:*** *'studies or data...in which there were interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (e.g., unphysiologic pathways of application) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for assessment and which is not convincing for an expert judgment.'*
4. ***Not assignable:*** *'studies or data....which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.).'*

The WFD TGD stresses that admissible data is not confined to GLP or guideline tests, but that all data may be used, irrespective of source, as long as it has been quality assessed and found valid. This is different than the recommendation given in the REACH TGD where standard or guideline tests are given preference (ECHA, 2011a).

The relevance of a study is primarily assessed by looking at the test endpoint. The EQS should protect the ecosystem and therefore it is important that the test endpoint is ecologically relevant. Relevant endpoints could be growth, reproduction, survival etc., whereas endpoints such as changes in enzyme induction or gene expression are not considered relevant.

After the data quality assessment has been made, the different data points are identified as either critical or supporting. Critical data are values for sensitive

species and endpoints, which are used as the basis for the EQS. Supporting data are values from tests that are for example not made with the most sensitive species or endpoint, values from tests that are difficult to interpret e.g. mesocosm studies), or are not fully reported (European Commision, 2011a). The distinction between critical and supporting data is only made when the AF method is used, because for the SSD approach all values are used in the construction of the SSD.

For many chemicals such as pesticides and some industrial chemicals a risk assessment may already have been made. For substances registered under REACH, it is recommended that the PNEC derived from the risk assessment process is adopted as the EQS. However, it is still important to collect any data that may have been presented after the REACH risk assessment was made, in order to take new evidence of more sensitive species or endpoints or toxic mode of action into account. Pesticides are reviewed under the Plant Protection Products (PPP) Directive (91/414/EEC) before they are put on the market. This review includes an assessment of freshwater ecotoxicity data, which is usually performed to GLP and follow standard test guidelines, and this data is therefore directly admissible for EQS derivation. However, since the risk assessment under the PPP Directive takes on a different approach than the risk assessment under REACH, an EQS cannot be directly derived from the dossier or the pesticide review report.

Special considerations for difficult substances

As mentioned in Chapter 1 of this thesis, many chemicals are difficult to handle in ecotoxicity tests due to physico-chemical properties such as sorption, volatilisation, ionisation, water solubility etc. This may not always be obvious from the result of an ecotoxicity test, and care should therefore be taken, when setting EQS for such substances. The TGD only provides limited guidance on how to take physico-chemical properties into consideration and only in cases where the substance is poorly soluble or where it is an ionising compound.

For substances with low solubility, it should be noted whether a solvent, emulsifier or a dispersant was used and at what concentration. For the test result to be regarded as reliable solvent controls must have been included in the test setup to verify that toxicity form the solvent has not occurred (European Commision, 2011a).

The toxicity of ionising compounds may depend on the pH of the test medium and therefore, the TGD recommends that attention should be paid to this in the

quality assessment of studies. For guideline or standard tests with ionisable compounds, the validity of the test should be reviewed if the pH is not within the required range specified for that test. Furthermore, if the pH of a test falls outside the range of naturally expected pH, the test should be rejected, regardless of test type. However, no clear directions are given to what exactly should be done if one or more pKa values are found for the compound, except that attention should be paid to possible pH effects on toxicity.

3 The influence of test conditions on ecotoxicity

The following chapter will first give an introduction to the background for and characteristics of guideline tests in order to point out some of the aspects of these tests that may influence the toxicity recorded herein.

3.1 Guideline tests

The majority of effect assessments made, whether they are part of a risk assessment or for EQS derivation, are based on test guideline, because these kind of data are often the only available or considered the most reliable (ECHA, 2011b; European Commision, 2011a). One of the major reasons why guideline tests are so often used is their promotion via the OECD chemicals programme established in 1971 (Diderich, 2007). The main objectives of the programme were to improve human health and the environment through improvement of chemical safety, to make chemical control and assessment more transparent and efficient, and prevent unnecessary distortions for the chemical industry and trade (Diderich, 2007). The basis of the programme was the concept of Mutual Acceptance of Data (MAD), i.e. that data generated under the OECD chemicals programme should be of a quality that could be accepted across borders and in different chemical assessment programmes (Diderich, 2007). Two principles were developed to ensure a harmonised data generation: the principles of Good Laboratory Practise (GLP) and the OECD test guidelines programme. The GLP principles are of a more general nature and addresses aspects of the structure and management of both laboratory and field studies, while the OECD test guidelines contain procedures for testing the chemical properties or effects of a substance (Rand, 1995).

Currently, there are 35 OECD test guidelines for testing the effect of chemicals on biotic systems using a large variety of species (aquatic and terrestrial), life stages and endpoints (OECD, 2013). Out of these 35 test guidelines 13 are tests for aquatic toxicity (no sediment present) (OECD, 2013). The OECD is not the only organisation that generates test guidelines for ecotoxicity testing. The International Organization for Standardization (ISO) has developed tests with many of the same species as recommended in the OECD guidelines, but the ISO standards are generally stricter in their requirements for the test system than the OECD guidelines. Thus, tests following an ISO standard will usually also fulfil the requirements of the corresponding OECD guideline. Other organisations, such as ASTM International (formerly known as the American Society for Testing and Materials) also develop tests guidelines. Although there are different

degrees of freedom with all test types, they all have the same tests principle: the organisms are exposed to a concentration gradient of the test compound for a given period of time and a response is observed in order to establish a concentration-response relationship.

A prerequisite for the establishment of a concentration-response relationship is that the test substance concentration is known and that it is kept (close to) constant over the whole incubation period. This is, among other things, facilitated by defining test conditions that are to be maintained throughout the test (see Box 1). This enhances the reproducibility of the test results and interferences from other types of stress, such as lack of nutrients, or pH or heat stress, are minimised because the test conditions are designed so they are optimal for the organism. Furthermore, constant test conditions during a test help prevent the substance from being transformed due to changes in the physico-chemicals conditions of the test solution, e.g. when the ionised fraction of an acid or base changes due to pH drift during incubation.

Box 1. General test conditions defined in OECD guideline tests for aquatic organisms exemplified by the OECD test guideline for *Lemna* sp. growth inhibition (OECD, 2006).

General term	**Example:** OECD test guideline 221. *Lemna* sp. Growth Inhibition Test
Temperature	24 ±2 C°
Light intensity	85-135 $\mu mol \cdot m^{-2} \cdot s^{-1}$
Light:dark rhythm	Continuous
Media	3 types to choose from, depending on species • SIS media (*L. minor*, pH 6.5) • 20X AAP (*L. gibba*, pH 7.5) • Steinberg (both species, pH 5.5)
pH	Depending on media type (see above)
Maximum pH drift	±1.5 pH unit (recommended, but not invalidating)
Oxygen content	Not relevant for *Lemna* sp.
Test duration	7 days
Feeding	Not relevant for *Lemna* sp.
Endpoint	Frond number, dry weight, frond area, fresh weight

SIS: Swedish Standards Institute

20X AAP: Algal Assay Procedure medium, 20 times stronger than original concentration.

Other measures to maintain a constant test substance concentration is to renew the medium at a certain time intervals or to establish flow-through conditions. This can make up for the loss of test substance due to hydrolysis or other degradation or transformation processes, but if the intervals between renewals are too long, the exposure concentration may still be fluctuating significantly. However, since this is assumed to be a more predictable fluctuation, it is often

compensated by using measured concentrations at renewals for calculation of a mean exposure concentration under the assumption of first order reaction kinetics. Measurement of the concentration is required when it is expected that the actual concentration varies more than 20% from the nominal concentration (OECD, 2006; OECD, 1998; OECD, 2000)

When the above measures (Box 1) are not enough to maintain the exposure concentration constant the "OECD guidance for aquatic testing of difficult substances" should be consulted (OECD, 2000). This document describes when a substance can be categorized as "difficult" and what measures or precautions can be taken to maintain a constant exposure concentration. This could for example be to condition the surface of the test vessel (silanisation for adsorbing substance) or to alter the pH of the test medium to a level which is consistent with the more toxic form of the compound (for ionising substances). The document also provides guidelines for calculation and expression of test results for difficult substances.

A very important part of a guideline test is to establish validity criteria and to validate the test system with reference compounds. Validity criteria are for example a minimum oxygen level, a maximum pH variation, or a minimum average growth rate throughout the test. If these criteria are not fulfilled, the test will be invalid. Tests with reference compounds are used prior to or in parallel with tests with the compound of interest. Achievement of an L(E)C50 value within a given range ensures that the test organisms are healthy and that the test system works properly.

3.2 The influence of test conditions

Measures made to maintain a constant test substance concentration throughout an OECD guideline test facilitate that the test results may be used for ranking, classification, and labelling of substances. However, since tests are always performed under the same conditions, which are optimised for growth and reproduction (e.g. at pH 7.5, continuous lighting, and 24 ± 2 °C in a test with *L. gibba*), important information may remain unrevealed if the substance is not tested under the conditions it exerts its highest toxicity. Furthermore, the physical and chemical conditions of guideline tests rarely reflect natural conditions, and therefore they may not be appropriate to use for risk assessment and derivation of EQS. Laskowski et al. (2010) reviewed the effects of several natural stressors on the toxicity of different chemicals on both terrestrial and aquatic animal species, and found that in approximately 50% of the 61 studies natural environmental

conditions had a significant effect on the toxicity. In the following the influence of changes in temperature, light and pH on the outcome of aquatic toxicity tests will briefly be described. Other test conditions may also influence the toxicity, but in the context of this thesis temperature, light, and pH were considered the most relevant concerning the extrapolations made to cover natural condition in EQS derivation.

3.2.1 Influence of temperature

Guideline tests are typically performed at a temperature that is in the high end of the organism's natural range. Keeping an optimal temperature ensures the optimal performance of the test organism, but it may also have consequences for the toxicity of the test substance. At higher temperatures the substance's tendency to escape the solution will be higher than at lower temperatures. Thus, for substances that are volatile from aqueous solutions (i.e. with a high Henry's Law constant), the concentration decline will be less at lower temperatures. Also the rate of hydrolysis is temperature dependent, and decreases with decreasing temperature (Harris, 1990). Hence, the predictions of chemical behaviour based on physico-chemical properties suggest that lowering the temperature could cause the concentration of the test compound to be maintained for a longer period than at higher temperatures. Furthermore, lower temperatures could affect the organism's uptake rate of the compound and/or slow down physiological processes that eliminate the compound or metabolise it (Li et al., 2011). On the contrary, a higher temperature could cause and increase in the metabolic oxygen demand of an organism and therefore also an increase in the toxicity of a chemical (Heugens et al., 2001). Consequently, it is not possible to predict the effects of changing the temperature from theoretical knowledge about chemistry and biology.

Conflicting results are found regarding the influence of temperature on the outcome of toxicity tests. For tests with fish and freshwater crustaceans it has been demonstrated that higher temperatures led to increased toxicity for metals. (Heugens et al., 2001; Heugens et al., 2003). However, for pesticides Heugens et al. (2001) reported that both positive, negative, and no correlation was found between temperature and toxicity. With regards to the effects of temperature on the toxicity of chemicals towards aquatic plants and algae, only one other study was found apart from the study contained in this thesis (Rosenkrantz et al., 2013a). Mayer et al. (1998) tested whether temperature had an effect on the toxicity of the compounds 3,4-dichloroaniline (weak base), 3,5-dichlorophenol (reference compound and weak acid), potassium dichromate (reference

compound), and atrazine (photosynthesis inhibiting herbicide) towards the freshwater green algae *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum)*. They found no direct temperature effect on toxicity, but an effect on toxicity was seen for interactions between light intensity and temperature because light saturation increases with temperature. In Rosenkrantz et al. (2013a) the toxicity of the four SUs mentioned in Chapter 1 towards *L. gibba* was measured at 15 and 24°C (i.e. the test at 24°C followed OECD test guideline 221 (OECD, 2006) for all parameters). A significant between the toxicity was only observed with flupyrsulfuron-methyl, where the EC50 increased with at factor 2 from 24 to 15°C. The EC50 for the other three compounds was not significantly different when tested at the two different temperatures. According to the OECD guideline, the biomass doubling time of the controls should be less than 2.5 days. The average doubling time of the controls in the study at 15°C was between 3.5 and 4.0 days, so the validity criteria was not met for this test. As a result of the slow growth the test was extended to last for 11 days in order to decrease the uncertainty associated with calculating effect concentrations from low frond numbers. However, since the plants were in exponential growth, the effect values calculated would have been the same if calculated at 7 days.

In conclusion, the above shows that even though many of the chemical and biological processes where temperature is a factor are known, it is impossible to predict the impact on toxicity from changing the temperature, and as also stated in Traas and Van Leeuwen (2007) there are no clear trends to be found in the ecotoxicological literature.

3.2.2 Influence of light

Light is also one of the important parameters of an ecotoxicity test, especially for plant tests but also for tests with animal species. Plant growth is dependent on light for photosynthesis, while the behaviour and activity of animals may be different under light and dark conditions. Therefore, both the influence of the duration of the photoperiod and the light intensity are relevant to investigate. Furthermore, the light conditions may also affect the test chemical by causing it to photodegrade faster at higher light intensities and longer photo periods. In theory, a highly photodegradable compound would be less toxic in a test with continuous lighting than in a test with a light:dark rhythm due to a higher photolysis and, thus, lower exposure concentrations (unless equally or more toxic degradation products are formed). In the following, focus will be on the influence

of photoperiod and light intensity on the toxicity of chemicals towards algae and plants.

In Rosenkrantz et al. (2013a) the tests made with the four SUs according to the OECD guideline was compared to one where a 12:12 hours light:dark rythm was introduced. We observed no clear trend in toxicity changes from this and only thifensulfuron-methyl showed a significant difference in EC50 compared to the EC50 from the standard test, i.e. a factor two increase with the application of a 12:12 hours light cycle.

As with temperature, the light intensity in a test should be within a given range. This is defined as 60-120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for freshwater algae (OECD, 2011b) and 85-135 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for the *Lemna* sp. test, and the level selected within the range should not vary more than ±15% throughout test. Both kinds of tests are carried out with continuous lighting. The light intensity is especially important in algal tests because the toxicity of the test compound may be affected if light saturation is not achieved (Cleuvers et al., 2002). In this study it was assumed that algae growing under light saturation would be more sensitive to that toxicant than under light limiting conditions. This assumption was supported by results showing that the toxicity of potassium dichromate towards *Scenedesmus subspicatus* increased with increased photon flux. Mayer et al. (1998) found the same tendency when testing the toxicity of 3,4-dichloroaniline, 3,5-dichlorophenol, and potassium dichromate towards *Selenastrum capricornutum.* Based on this, they recommended that the light intensity in tests with green algae should be at least 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$in order to minimise the variability that was observed at lower light intensities. No studies have been found that investigate the impact of light intensity on toxicity towards *Lemna* sp., but one study was found that investigates the toxicity of atrazine towards the submersed macrophyte *Elodea canadensis* in the dark and at two different light intensities, using shoot-length and dry shoot weight a end-points (Brain et al., 2012). Consistent with the algal studies, this study showed that the toxicity increased with increasing light intensity. Thus, it seems that in most cases, choosing a light intensity in the high end of the range given in the guidelines will result in higher toxicities than at lower intensities. However, none of the chemicals tested above are photodegradable, and this property could have an impact on the toxicity, unless equally or more toxic degradation products are produced.

3.2.3 Influence of pH

There are large variations in the recommendations of media pH between the different OECD test guidelines. Some require a start pH value adjusted to within ±0.1 pH unit (OECD, 2006), while others are just required to be in the range of pH 6-9 and should not be adjusted unless it is outside the range (OECD, 1998; OECD, 2011a; OECD, 2004). There is also often a recommendation for the maximum drift allowed, which is usually 1.5 pH units during a test, but in some cases only 0.5 units are allowed, either as a general rule or when electrolytes or metals are tested (OECD, 2012; OECD, 2011b).

The pH of the tests solution is important for several reasons. For substances that hydrolyse, changes in the pH of the solution may accelerate or slow down the process, such as is the case for many SUs, e.g. flupyrsulfuron and rimsulfuron (Table 1). The consequence of this is that the test compound may disappear from the solution and degradation products are formed. Thus the exposure concentration of the tested compound is no longer constant and measurements must be made to determine the actual concentration. Due to an often limited number of chemical analysis performed, the exposure concentrations are then calculated as an average of the measured concentrations at the beginning and end of the test period.

For substances such as electrolytes the pH of the solution can be of major importance for the toxicity. The uptake and toxicity of acids and bases are pH-sensitive because the pH of the medium affects the speciation of the compound. According to the theory about uptake of chemicals into cells and ion trapping, it is primarily the neutral form of the electrolyte that can penetrate the cell membrane, and enter the cell to cause toxicity, whereas the ionised and more water soluble fraction, will mainly stay outside the cell (Trapp, 2000; Trapp, 2004; Escher and Hermens, 2004; Neuwoehner and Escher, 2011). The fraction of the neutral compound, f_n, present in the test solution at a given pH can be calculated with the following equation, which is based on the Henderson-Hasselbalch equation (Atkins, 1990):

$$f_n = \frac{1}{1 + 10^{\alpha(pH - pKa)}}$$

where α is -1 for acids and +1 for bases.

Accordingly, tests with electrolytes may result in different toxicities towards the same organism at different pH values, and it is expected that toxicity will be higher for acids at low pH and for bases at high pH. The pH dependent toxicity of chemicals has been studied extensively, and was recently reviewed by Rendal et al. (2011). In this review, 47 studies were found that tested the toxicity of acids at different pH level and 28 studies that tests the toxicity of bases at different pH levels. The review showed that the exposure pH affected the toxicity of the compounds, in some cases with up to a factor 100 or more. They also showed that the factor of change increased with log Kow (positive correlation for both acids and bases, but only statistically significant for acids) and that is was higher when an ion-trap effect took place. In Rosenkrantz al. (2013a), the toxicity of the four SUs (Table 1) was tested at pH 6, 7.5 (OECD guideline test) and 9, to examine whether the pH would also have an effect on the toxicity towards *L. gibba*. For all four SUs we observed a clear trend of higher EC values (lower toxicity) with higher pH, with the effect being most pronounced for flupyrsulfuron-methyl and thifensulfuron-methyl (Figure 4). The EC50 increased between 2.2 and 10 times for the four SUs when pH was increased from 6 to 9.

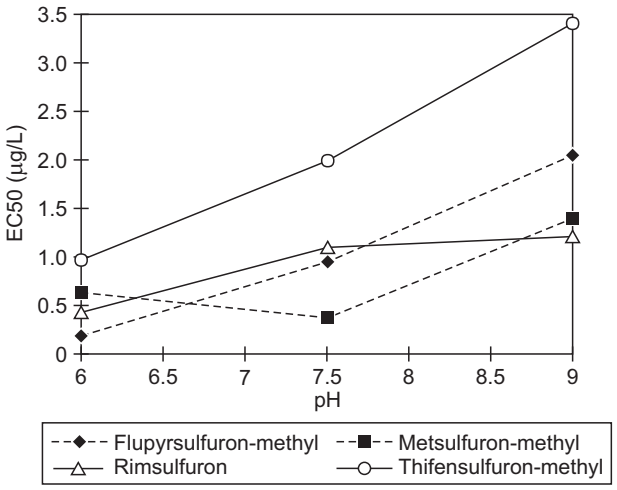


Figure 4. The EC50 for the four sulfonylurea herbicides as a function of the initial pH of the test solution (Rosenkrantz et al., 2013a)

In order to maintain the pH on the initial level, synthetic buffers were added in the tests at pH 6.0 and 9.0 (10 mM MES and TRIS, respectively) and this was sufficient to maintain the pH stable to within ± 0.3 pH units. In the guideline tests the only buffer used was the $NaHCO_3$ recommended in the media recipe. Here, a significant drift of +1.2-1.6 pH units was seen in all four tests. An attempt was made to minimize the drift by adding MOPS buffer (unpublished results), but this buffer could not counteract the pH drift although it has a range of pH 6.5-7.9. In

Figure 5, the estimated concentrations-response curves from the tests are seen, and for the tests at pH 9.0, it is seen that the growth rate of the controls are lower than in the tests at pH 6.0 and 7.5 (initial pH). Doubling times for the controls here were 2.3-2.4 days as compared with 1.3-1.7 days in tests at lower pH values, suggesting that the TRIS buffer used in the pH 9.0 tests could have an inhibiting effect on the plants. However all doubling times was below the validity criteria, which is $T_2 < 2.5$ days, thus fulfilling this criterion

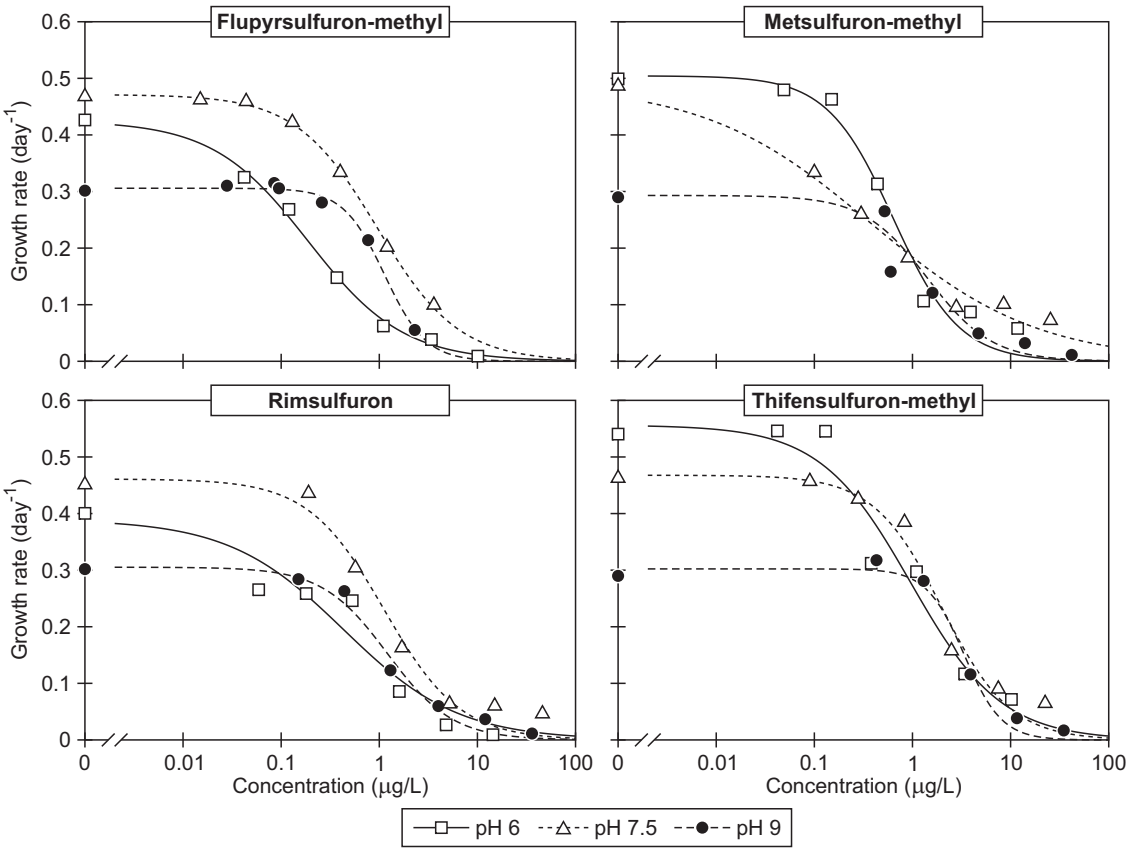


Figure 5. Concentration-response curves for the four sulfonylurea herbicides at pH 6 (□), 7.5 (•) and 9(Δ) (Rosenkrantz et al., 2013a).

3.3 Maintaining constant test conditions

Before commencement of the tests presented in Rosenkrantz et al. (2013a) the physico-chemical properties of the four SUs were evaluated and because some of them had short hydrolysis half-lives, it was decided to do static renewal tests with media renewal two times during the test (every 2-3 days). Concentrations were measured at the beginning and end of each renewal period, and the chemical analyses showed large concentration variations, both between nominal and measured start concentrations and between measured concentrations at the

start and end of renewal periods. An explanation to the differences found between nominal and measured concentrations could be that preparation of test solutions with very low concentrations can be difficult to do because it is necessary to weigh out very small amounts of test substance in order to avoid the production of unnecessarily large volumes. Differences between measured concentrations at the beginning and end of renewal periods were probably caused by hydrolysis of the compounds during incubation. The loss of test substance was found to be largest for flupyrsulfuron-methyl and rimsulfuron, which is in good agreement with the hydrolysis half-lives (DT50) presented in Table 1. Moreover, it was observed that the dissipation of flupyrsulfuron-methyl and rimsulfuron was less at pH 6 than at pH 7.5 and 9, which also fit well with the physico-chemical properties of the compounds. In conclusion, the measures to maintain tests substance concentrations given in the difficult substances guideline (OECD, 2000) were followed, but still large variation were measured, and a time-weighted mean of the test concentrations was necessary for the expression of the results.

A number of the above mentioned difficulties in maintaining constant exposure concentrations throughout the incubation period are also time-dependent. In an attempt to overcome some of the difficulties Rosenkrantz et al. (2013c) presented a method to examine the toxicity towards algae in a short-term test. The hypothesis of this study was that by reducing the duration of the exposure from 72 hours to 2 hours, changes in test concentration and/or bioavailability of the tested compounds would be minimised. It was concluded from the study that, although the test resulted in effect values similar to those from 72-hours guideline tests for 3,5-dichlorophenol, atrazine and terbuthylazine, the test was not able to detect toxic responses for all types of compounds. For the SUs, it was not possible to establish a concentration-response relationship using this test, which by Rosenkrantz et al. (2013c) was suggested to be due to their mode of action as inhibitors of cell division. It was found that the incubation period in this test system was most likely too short for the effect to become measurable. Therefore, the conclusion of the study was that the method provides a good alternative or supplement to guideline tests for compounds which disappear rapidly from the solution and for which toxic effects occur relatively fast e.g. as a result of a fast uptake or a specific acute mode-of-action in algae.

4 The influence of exposure duration

In the previous chapter it was described how deviations of some of the physical and chemical test conditions from test guidelines could affect the result of a test. Another well-defined parameter of the OECD test guidelines (and others) is the test duration, which is set so that it describes effects (acute or chronic) from continuous exposure. However, the exposure to for example pesticides rarely occurs as a continuous discharge but rather as a pulse of a shorter duration. Therefore, the following will give a description of the nature of chemical pulses and effects of pulse exposure in the laboratory, in order to be able compare the effects of the two exposure types. Focus will be on pulse exposure of pesticides, although it is recognised that other types of discharges (e.g. sewer overflows during heavy rain events) may also be of a pulsed nature.

4.1 Pulse exposure

Aquatic organisms in nature will more likely be exposed to pesticides as one or several consecutive pulses than continuously exposed. A pulse may occur as runoff during a rain event after spraying and/or via spray drift into a waterbody near the spraying field. The duration of a pulse is reported to last anywhere from a few minutes to several hours, depending on agriculture practice, waterbody characteristics, climate, and chemical properties of the compound in question. Furthermore, pulses may occur as both single and repeated pulses, and with different frequencies, depending on weather conditions during and after application and frequency of spraying (Cedergreen et al., 2005; Handy, 1994; Reinert et al., 2002; Styczen et al., 2003).

In addition to the pulse exposure scenario described above, pulse exposure may also occur, both in the field and in the laboratory, as a result of fast transformation reactions or phase distributions after application, with a resulting fast decrease in exposure concentrations (Reinert et al., 2002; Hommen et al., 2010) Such transformation reactions could be degradation by hydrolysis, photolysis, or biodegradation. Also phase distribution processes like sorption or evaporation may rapidly remove the test compound from the test solution, making it unavailable for uptake by the test organism.

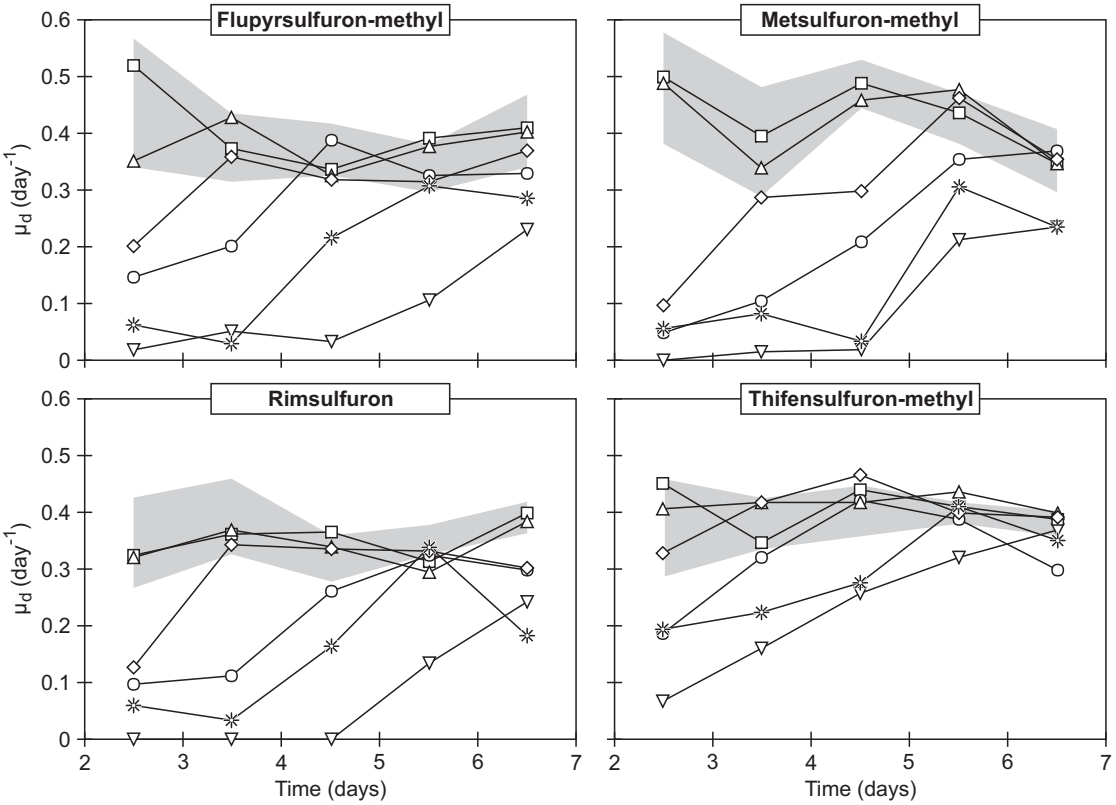
4.2 Ecotoxicological effects from pulse exposure

Pulse exposure studies of effects of pesticides towards freshwater invertebrates are quite abundant in literature (e.g. Andersen et al., 2006; Cold and Forbes, 2004; Forbes and Cold, 2005; Heckmann and Friberg, 2005; Mugni et al., 2011; Naddy et al., 2000; Naddy and Klaine, 2001; Parsons and Surgeoner, 1991; Reinert et al., 2002). These and other studies report effects from single and repeated pulses, which was seen both during the exposure and as delayed effects occurring after the exposure. Different types of effects was observed, such as instant immobilisation and death, and delayed effects observed as increased mortality, decreased reproduction, and delayed development.

The literature about pulse exposure effects on aquatic plants and algae is much scarcer. Cedergreen et al. (2005) compared the effects of 3-hour pulses with long term exposure (4 or 7 days) of six herbicides to *Lemna minor*. They found that, for imazamox, metsulfuron-methyl, propyzamide and pendimethalin, a 3-hours pulse had the same effect as a 4-days exposure but at a concentration that was 10 times higher. The same trend was found for terbuthylazine and diquat but at a 100 times higher concentration. After the pulse exposure the recovery of the plants was studied, and it was observed that plants exposed to photosynthesis inhibitors, such as terbuthylazine and diquat, recovered within 24 hours, while plants exposed to ALS inhibitors (imazamox and metsulfuron-methyl) and microtubule assembly inhibitors (propyzamide and pendimethalin) took up to 4 days to recover. Valloton et al. (2008; 2009) investigated how algal growth was affected by single and repeated pulses of different duration, frequency and isoproturon (photosynthesis inhibitor) concentrations. It was found that even though all treatments caused an inhibition of the algal growth, the algae recovered relatively fast. However, an initial inhibition of growth will still cause a decrease in the total biomass production. In another study, Belgers et al. (2011) exposed the rooted submersed macrophyte *Myriophyllum spicatum* to metsulfuron-methyl for different periods (1-21 days) and concentrations (0.1-21000 ng/L) so that each exposure scenario had the same time-weighted average (TWA) concentration. Their conclusion was that large difference in effect values of the different exposure scenarios were seen if the effect values (e.g. EC50) were calculated from the exposure concentrations only, while no significant difference was found if the effect values were based on the TWA concentrations. In other word, Belgers et al. (2011) found that for *M. spicatum*, if seen over a longer period, the effect of a high concentration pulse was similar to the effect of a continuous exposure to a low concentration. However, it should be mentioned

that, compared to *Lemna* sp., *M. spicatum* is rather slow-growing, and an experiment with duckweed did indeed not come to the same conclusion. Boxall et al (2013) tested the effect of repeated two- and four-day pulses of isoproturon, metsulfuron-methyl, and pentachlorophenol (PCP) on *L. minor* for a total observation period of seven weeks. The results of the pulse tests was compared with the result of the continuous exposure tests, and it was found that for isoproturon, the response was lower in the pulse tests than for in the continuous tests, while for metsulfuron-methyl it was similar and for PCP it was higher. Therefore, the authors concluded that a simple time-weighted average approach may not provide an accurate prediction of pulse exposure effects, and instead they suggest development of mechanistic models for prediction of effects from time-varying exposures.

Finally, the study described in Rosenkrantz et al. (2013b) compared the effects of a 24-hour herbicide pulse on the growth of *L. gibba* with effects observed in a test with continuous exposure (OECD guideline test). The test was made with the four SUs mentioned in Chapter 1, and the plants were exposed to a gradient of each substance for 24 hours and then observed in a 6-days post-exposure period (total test duration 7 days) in herbicide-free media. The results showed that concentrations slightly above the EC50 values from the OECD tests initially caused a lower growth in the pulse exposure tests, but that the growth rate of these plants reached the level of the controls during the post-exposure period. Plants exposed to the highest concentration of flupyrsulfuron-methyl, metsulfuron-methyl, and rimsulfuron did not reach the growth rate level of the controls within the post exposure period, while all treatment groups reached the level of the controls for thifensulfuron-methyl. This is visualised in Figure 6, which shows the day-to-day growth rates. To compare the effects of the pulse exposure with those of the continuous exposure EC50 values were calculated based on biomass yield instead of growth rates, because the growth rates in the pulse tests were not constant over the whole period. The comparison showed that the pulse exposure tests resulted in 2-6 times higher EC50-values than the continuous exposure tests, and it is concluded that with the approach of this study, effects of the two exposure types can be compared without using time-weighted average concentrations. However, the approach still does not take account of the observed recovery, and as mentioned in Boxall et al. (2013), this could possibly be done by applying a mechanistic model calibrated with test results.



	Control	Pulse concentration (µg/L)					
		□	△	◇	○	✳	▽
Flupyrsulfuron-methyl		0.02	0.07	0.27	1.1	4.3	17
Metsulfuron-methyl		0.03	0.12	0.46	1.8	7.4	29
Rimsulfuron		0.04	0.16	0.62	2.5	9.9	40
Thifensulfuron-methyl		0.03	0.11	0.44	1.8	7.1	28

Figure 6. The day-to-day growth rates (day^{-1}) for each concentration of four sulfonylurea herbicides in the post exposure period after a 24-hours exposure. The observations from day 1-2 of the post exposure period are omitted from the plots because of a general observation of a lag phase following the pulse exposure (Rosenkrantz et al., 2013b).

5 Discussion

5.1 Test conditions

In chapter 3 a description of the general characteristics of OECD guidelines for testing aquatic toxicity was given, and this was used as a starting point for investigations about the influence of physico-chemical test conditions on the results of toxicity tests. For temperature and light no general trends were found in literature or in the experiments carried out as to whether an increase or decrease would cause a certain change in toxicity. However, it was shown that both can have a statistically significant impact on toxicity. As no correlations between toxicity and temperature and light conditions were found, it seems acceptable to use the present conditions in current guideline tests as the basis for derivation of EQS or in risk assessments. On the other hand, it could be argued that more environmental realism would be added to the test result if a suitable light:dark rhythm was introduced along with a more environmentally realistic light intensity and temperature. The trade-off of these changes would most likely be an increase in the variation of the response and thereby reduced reproducibility of the tests. Moreover, in Rosenkrantz et al. (2013a) lowering the temperature made it necessary to extend the test duration from 7 to 11 days and hence, increasing the environmental realism of tests would also mean an increase in the costs of testing.

For toxicity tests with electrolytes at different pH levels a general trend in toxicity change was seen. This is supported by both the present study and scientific literature, and cannot be considered a new finding. Thus also OECD's "difficult substances guideline" state that *"The definitive test should be conducted at a pH consistent with the more toxic form of the substance whilst remaining within the range required to maintain the health of the control organisms."* (OECD, 2000). This was further explored by Rendal (2013), who, based on the review of available literature and own studies, recommends that if an acid has a pKa value in the range 3-8 it should be tested at pH 6 and if the pKa is in the range 8-10 it should be tested at pH=pKa-2. Likewise for bases, if they have a pKa in the range 7-12 they should be tested at pH 9, and if pKa is in the range 5-7, they should be tested at pH=pKa+2. Doing this would in most cases secure that the most toxic form of the substance is tested. The current study adds to this recommendation that careful inspection of the physico-chemical properties of the test compound should be done prior to testing, and the test conditions

chosen according to the projected use of the data, while still obtaining high reproducibility and sensitivity.

The original purpose of performing guideline tests was to enable ranking and classification of chemicals, and this can only be done if a high degree of reproducibility is achieved. This requires that the test organism is exposed to the toxicant continuously and at a constant level throughout the test. This applies even if this level has to be maintained artificially and without taking the inherent properties of the compound into account. It could, however, be questioned whether it is reasonable to use the result of such a test as the basis for an EQS derivation, since it entails little environmental realism. The EQS is set in order to protect the environment, and therefore its basis should also, to the extent possible for a laboratory test, reflect natural conditions. Substances, such as flupyrsulfuron-methyl and rimsulfuron, would probably undergo hydrolyse rather quickly (within days) after discharge (Table 1). Therefore a toxicity test with one of these compounds, where the concentration is artificially maintained constant, would possibly overestimate the toxicity, and consequently the resulting EQS would be too strict. Furthermore, as it is today, the WFD does not take recovery of the test organism into consideration when EQS are derived. Performing a toxicity test where the test substance decreases or even disappears before the test ends would automatically incorporate a (at least initial) recovery of the test organism, and therefore better reflect the environmental fate processes in the result. Hence, it is obvious that using the same test for two different purposes (classification vs. environmental protection) could cause problems. It is therefore important that the intended use of the test should be clarified before the test is performed.

As described in Chapter 3.3 it proved difficult to maintain constant test concentrations in the *Lemna* sp. tests that formed the basis in Rosenkrantz et al., (2013a). When this occurs, the traditional path to follow, in order to live up to the MAD requirements, is to consult the difficult substances guideline (OECD, 2000). The result of this would be a test where the exposure concentrations are constant, but at the expense of lowering the environmental realism. Instead of consulting the difficult substances guideline (OECD, 2000), another option could be to perform a different type of test, and thereby get additional information on the tests substance. This was the objective of Rosenkrantz et al. (2013c), where a method to test the toxicity towards algae in a short test was presented and evaluated. Supplemental information from such a test could prove important to the EQS derivation. However, since it is not a guideline test, results from this

test t would probably be assigned a quality code 2 or 3 on the Klimisch scale (Klimisch et al., 1997) and may in practice be excluded from the dataset forming the basis for the EQS derivation. This build-in practice of favouring guideline test for EQS derivation and risk assessment may cause data that could influence the resulting EQS to be left out of the assessment. This was also pointed out by Ågerstrand et al. (2011), who suggest a further development of the reporting and evaluation criteria of data to allow the inclusion of non-guideline test data in risk assessments.

5.2 Pulse exposures

Chapter 4 reported that several studies have shown that pulsed exposure to chemicals can have both acute and chronic effects on organisms. Most of these studies are made with freshwater invertebrates but for herbicides these organisms are not as relevant as aquatic plants and algae. Only a few studies have been published that investigate effects of pulse exposures on plants and algae and to add to the knowledge in this area, Rosenkrantz et al. (2013b) investigated the effect of a 24-hours pulse of each of the SUs towards *L. gibba*. According to the TGD (European Commission, 2011a) there is a factor 10 between the AA-EQS and the MAC-EQS, when only data for short-term tests are available (see Table 3), i.e. safe pulse exposure concentrations are allowed to be 10 times higher than the average exposure concentration considered to be safe. However, it was shown that the pulse tests resulted in EC50 concentrations that were only 2-6 times higher than those obtained in a guideline test (7 days), and not the expected factor of 10 or more. Therefore, the approach presented in this paper indicates that for SUs, an AF of 100 is too low and hence, the resulting MAC-EQS will be under-protective. In the light of this, automatically applying a factor 10 reduction (from 1000 to 100) in AF for MAC-EQS derivation for all compounds, may be questioned, and we therefore suggest to investigate experimentally, as for example in the present study, what the AF should be. However, this evaluation would probably be more relevant for certain groups of compounds (e.g. pesticides) and the relevance of performing such a test should be evaluated beforehand, based on chemical properties and expected use pattern.

The TGD (European Commission, 2011a) applies an AF of 100 for derivation of a MAC-EQS, but the rationale for choosing this value has not been found in any of the publically available guidance documents (ECHA, 2008; European Commision, 2011a; Lepper, 2002). However, in a report from the Danish Environmental Protection Agency (Tørsløv et al,. 2002), the following is

deliberations are stated in a section about the establishment of a MAC-EQS: a study is described where the ratio between the acute (i.e. E(L)C50 values) and chronic (i.e. NOEC values) toxicity (a/c-ratio) is calculated for 72 substances comprising both pesticides, metal compounds, and other, both organic and inorganic, compounds. A/c-ratios were found between 0.126 and 1290, with a median of 9.0. This report states that the TGD operates with an implicit factor of 10 between acute and chronic toxicity because the AF used for derivation of the $PNEC_{intermittent}$ (corresponding to the MAC-EQS) is 100 and the AF used for the general PNEC (corresponding to the AA-EQS) is 1000. However this statement is incorrect since the availability of chronic toxicity data will result in an AF of 100 or less (see Table 3). An AF of 1000 is used when only data from short-term tests showing acute effects are available, and hence, the indicated relationship between an a/c ratio of 9 and the implicit factor of 10 found when comparing AFs seems to be irrelevant.

While the test approach described in Rosenkrantz et al. (2013b) provides a method to experimentally compare the effects of pulse exposures with effects found in guideline tests, it does not account for the potential recovery of the organism. The same holds true for the methods used to derive EQS under the WFD (European Commision, 2011a; Hommen et al., 2010). In Rosenkrantz et al. (2013b) it was shown that, based on the growth rate (Figure 6), the plants are (in the process of) recovering and although the pulse does cause a loss in biomass production, the growth rates in all treatment groups, for all four SUs, are almost at the level of the controls within a week for all SUs at all tested concentrations. Hence, the EC50 values found mainly reflects the loss of biomass production. Nevertheless, the pulse effect should not be neglected just because the organism recovers. In Andersen et al. (2006) it was demonstrated that *Daphnia magna* revealed chronic effects in the post exposure period even though they recovered mobility after pulse exposure to acetylcholinesterase inhibitors. Conversely, for macrophytes, a decrease in biomass production or growth could result in alterations in the ecosystem composition where a reduction (or the complete loss) of species that are more sensitive than the one tested could give room for more resistant or invasive species. In summary, it is therefore recommended, that instead of basing the MAC-EQS on tests with continuous exposure and application of somewhat arbitrary AFs, it should either be based on experimental comparisons of effects, such as in Rosenkrantz et al. (2013b) or on mechanistic models that estimates the effects of pulsed exposure and subsequent recovery (Ashauer et al., 2006; Boxall et al., 2013).

5.3 EQS derivation – the case of sulfonylureas

When comparing the ecotoxicity values (Table 2) given in the review reports (European Commission, 2000b; European Commission 2001a; European Commission, 2001b; EFSA, 2005), test with laboratory cultured algae gave EC50 values that were in the range of 1.5-260 times higher than the values from *Lemna* sp. tests. Hence, the possibility of natural algal species or communities being equally or more sensitive could not be disregarded and was considered relevant to investigate. Such an investigation was possible using the method described in Rosenkrantz et al. (2013c). Tests were performed with natural algal communities from Lake Fure and the four SUs were used as test substances along with atrazine. The results showed that, for atrazine, using this method allowed the examination of the toxicity towards a natural algal community, and that the sensitivity of the natural algae was similar to that of the laboratory-grown *P. subcapitata*. However, for the SUs the method was not feasible as no concentration-response relationship could be established due to the mode of action of the SUs (see Chapter 3.3). Therefore, inclusion of data from this study (Rosenkrantz et al., 2013c) does not alter the assumption that when deriving EQS for SUs the most sensitive species tested is *Lemna* sp. and the AF should be chosen accordingly.

One way to evaluate the implications of the findings in Rosenkrantz et al. (2013a) is to look at the resulting EQS. Since the compounds are registered plant protection products a lot of data is available in the review reports (European Commission 2000b; European Commission 2001b; European Commission 2001a; EFSA 2005). The amount of additional data from the scientific literature is, however, highly variable for the four SUs. Metsulfuron-methyl is used extensively as representative for the ALS inhibitors, while the other three compounds are studied more sparingly or not at all in the scientific literature when it comes to ecotoxicology studies (see Table 2 for the values found). Nevertheless, based on the amount of data available and because the most sensitive species has (most likely) been tested, an AF of 10 can be used for all four compounds for the derivation of the AA-EQS. The tentative EQS calculated from the EC10 values found in Rosenkrantz et al. (2013a) are given in Table 4 together with proposed AA- EQS for Sweden (KemI, 2008).

Table 4. Tentative Annual-Average Environmental Quality Standards (AA-EQS) estimated from EC10 at different pH levels (Rosenkrantz et al., 2013a) using an assessment factor of 10. Proposed AA-EQS from Sweden (KemI, 2008) are given in the bottom row.

		Flupyrsulfuron-methyl	**Metsulfuron-methyl**	**Rimsulfuron**	**Thifensulfuron-methyl**
		ng/L	ng/L	ng/L	ng/L
AA-EQS	pH 6	1.6	12	2.2	9.2
AA-EQS	pH 7.5	12	27	16	34
AA-EQS	pH 9	72	27	23	130
AA-EQS	KemI	50	20	10	50

The AA-EQS at pH 7.5 are comparable to the values from KemI, except for the one from flupyrsulfuron-methyl. This value is based on a 14-days static test with biomass as endpoint (KemI, 2008), and it is possible that most of the test substance have hydrolysed during the test, and thereby allowed the plants to recover to a certain degree. However, as already stated in Chapter 3, the difference between the effects at low and high pH is significant, and again the importance of evaluating the test substance before testing is stressed.

In general, the EQS are very low for the SUs, and measuring concentrations this low in environmental samples would be difficult if not impossible. However, due to the use of SUs as herbicides for field application, they are more likely to be discharged to the environment in pulses than as a continuous discharge. Hence, it could be questioned if it even makes sense to derive an AA-EQS for compounds like these. In response to this, it is recommended that in addition to the evaluation of the physico-chemical properties of the substance prior to choosing tests design, the use pattern of the substance should also be included. Thus, for pesticides, more focus should be on deriving MAC-EQS based on tests that describe the effects of single and repeated pulses, compared to only describing effects of continuous exposure.

The findings in Rosenkrantz et al. (2013b) are not directly usable for derivation of MAC-EQS with the current method, but they do indicate, that the method could be improved by basing the MAC-EQS on pulse tests and also considering the recovery of the organism.

6 Conclusion

Derivation of an environmental quality standard will often be based on results from ecotoxicological tests performed according to internationally approved guidelines, such as the OECD test guidelines. These guidelines were originally developed for classification and hazard ranking of chemicals. Toxicity in these tests are measured as a function of continuous chemical exposure under test conditions that rarely reflect natural conditions, but aims at improving test reproducibility. The aim of this thesis was to investigate whether environmental quality standards derived on the basis of guideline tests will be sufficiently protective of the environment.

This study showed that changing the physical and chemical test conditions influences the toxicity of sulfonylurea herbicides towards the macrophyte *L. gibba.* Thus, there may be a risk of over- or under-estimating the toxicity if only tests performed according to approved guidelines are available. Consequently, it is recommended to carefully inspect the physico-chemical properties of the test compound prior to testing and to design the tests accordingly. The test should be designed in a way that reveals the most toxic form of the compound, while keeping the test conditions within an environmentally realistic range.

With regards to pulse exposure, it was shown that it is possible to experimentally determine the difference between effects from a pulse and from a continuous exposure for *L. gibba*. Differences between effects from the two exposure types were demonstrated, indicating that the current approach for derivation of Maximum-Allowable-Concentration Environmental Quality Standard (MAC-EQS) may be under-protective for some substances. Furthermore, it was also pointed out, that protection from pulsed discharges should not be based on the results of tests with a continuous exposure, even if they are short-term tests. Therefore, it is suggested that the derivation of a MAC-EQS should be based on pulse tests and possibly also modelling, and the recovery of the organism should be considered.

The overall conclusion of this thesis is that under the current approach for derivation of EQS, there will be cases where basing the value on results from guideline tests will not be appropriately protective of the environment. Bringing more environmental realism into the testing by designing tests according to the physico-chemical properties and taking the use pattern of the compound into consideration would probably result in a better estimation of the EQS.

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Papers

Paper I: **Rosenkrantz, R.T.**, Cedergreen, N., Baun, A., Kusk, K.O. 2013a. Influence of pH, light cycle, and temperature on ecotoxicity of four sulfonylurea herbicides towards *Lemna gibba*. *Ecotoxicology*, 22 (1), 33-41.

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