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Effects of chemicals on waste water treatment: Final validation study of the protozoan activated sludge test to establish an OECD Test Guideline

von

Viktoria Pauli, Wilfried Pauli
Institute of Biology, Free University of Berlin, Berlin

Free University of Berlin
Institute of Biology
Altensteinstr. 4
14195 Berlin

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Autor(en) (Name, Vorname)	Pauli, Viktoria; Pauli, Wilfried
Durchführende Institution (Name, Anschrift)	Freie Universität Berlin Institut für Biologie Altensteinstr. 4 14195 Berlin
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Author(s) (Family Name, First Name)	Pauli, Viktoria; Pauli, Wilfried
Performing Organisation (Name, Address)	Free University of Berlin, Institute of Biology Altenstr. 4, 14195 Berlin
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Kurzbeschreibung

Protozoen bilden ein wesentliches Glied in der Abwasserreinigung. Von ihrer Filtrationsleistung hängt ab, ob und inwieweit Einzelbakterien ausfiltriert werden und damit die eigentliche Klärung des Abwassers erfolgt. Bei der Risikobewertung von Chemikalien in Kläranlagen ist diese trophisch und funktionell bedeutende Organismengruppe bislang allerdings nicht berücksichtigt. Zwischen 2012 und 2014 wurde ein Ringtest mit dem Ziel durchgeführt, den "Belebtschlamm Protozoentest" abschließend zu validieren und anhand der Ergebnisse einen OECD-Testrichtlinienentwurf zu erstellen.

Der Ringtest wurde im Auftrag des Umweltbundesamtes von der Freien Universität Berlin organisiert und koordiniert. Die Ringstudie wurde anhand von fünf Referenzsubstanzen in fünf Labors durchgeführt. Alle Teilnehmer waren in der Lage, Konzentrations-Wirkungskurven für alle Testchemikalien zu ermitteln. Hierbei wurde pro Testlauf die Wirkung von mindestens fünf Konzentrationen mit jeweils drei Replikaten erfasst.

Von den Teilnehmern wurden 31 Testläufe durchgeführt, 26 davon entsprachen den im Testprotokoll vorgesehenen Validitätskriterien. Für 25 Testläufe konnten EC_{50}-Werte ermittelt werden. In einem Fall konnte keine Wirkung nachgewiesen werden. Die ermittelten EC_{50}-Werte umspannen einen Wirkbereich von mehr als vier Zehnerpotenzen. Im Mittel weichen die in den veschiedenen Labors ermittelten EC-Werte um den Faktor von 2,6 mit einem Variations-koeffizienten von 36% voneinander ab. Dabei liegen die Extremwerte des Interlaborvergleichs bei einer Schwankungsbreite von 2- bis 3,45-fach und einem Variationskoeffizienten von 27% bis 49%.

Abstract

As a constitutive group within the microbial food web, protozoa play an important role in sewage treatment. Their feeding on bacteria improves the treatment, resulting in higher transparency, i.e. lower organic loads in the effluent. Although vital for the functioning of sewage treatment plants, protozoa are presently not covered by the currently standardized ecotoxicological test systems.

Between 2012 and 2014 this final ring test took place with the objective to formally validate the 'protozoan activated sludge test'. It was coordinated by the Free University of Berlin on behalf of, and with funding from the German Environmental Agency (UBA). Five laboratories participated in this collaborative validation study.

In all labs tests were performed with five model chemicals using at least five concentrations steps (three replicates per step). In total, 31 test runs were performed. EC_{50}-values could be calculated for 25 out of 26 valid test runs. In one case no chemical effect could be observed. EC50s cover more than 4 orders of magnitude. They vary across laboratories on average by a factor of 2.60 with a mean coefficient of variation (CV) of 36%. The extreme values of the inter-lab EC_{50}-deviation are going from 2-fold to 3.45-fold with coefficients of variation in the range of 27% to 49%.

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Abbreviations

CERI	Chemicals Evaluation and Research Institute
CV	coefficient of variation
DMSO	dimethyl sulfoxide
EC	effective concentration
E.coli	*Escherichia coli*
FU	Free University of Berlin
GLP	good laboratory practice
LOEC	lowest observe effect concentration
$logP_{ow}$	$logarithm_{10}$ of the octanol-water partition coefficient
NOEC	no observed effect concentration
OD	optical density
SOP	Standard Operating Procedure
STP	sewage treatment plant
UBA	Umweltbundesamt (German Environment Agency)
3,5-DCP	3,5-dichlorophenol

Zusammenfassung

EINLEITUNG

Erkenntnisstand

Protozoen bilden ein wesentliches Glied in der Abwasserreinigung. Von ihrer Filtrationsleistung hängt ab, ob und inwieweit Einzelbakterien ausfiltriert werden und damit die eigentliche Klärung des Abwassers erfolgt. Von offizieller Seite wird deshalb schon seit mehreren Jahren auf die Notwendigkeit eines Tests verwiesen, der die funktionale Integrität von Protozoen in Belebtschlämmen erfasst und als Grundlage zur Risikobewertung von Chemikalien dienen kann (z.B. TGD, 1998, 2003; ECHA, 2014).

Im Rahmen eines vom Umweltbundesamt geförderten F.&.E.-Vorhabens (FKZ 201 67 402) wurde in den Jahren zwischen 2002 und 2005 an der Freien Universität Berlin der erste Schritt zur Erfüllung der behördlichen Vorgaben unternommen und ein Test entwickelt, der die Filtrationsleistung als funktional entscheidenden Parameter der Protozoenbiozönose von Belebtschlämmen erfasst (Pauli & Poka, 2005). Hierbei werden Belebtschlammproben über einen Zeitraum von 22 Stunden bei einer Temperatur von 22°C verschiedenen Konzentrationen der Testchemikalie exponiert. Der Test findet unter statischen Bedingungen mit 2 mL-Proben in kleinen, septumverschlossenen Glasröhrchen statt. Zu Beginn des Tests erhalten alle Proben Bakterien als Nährsubstrat. Um die Phagozytose sichtbar zu machen, wird am Anfang und am Ende die optische Dichte der Proben photometrisch gemessen. Während gehemmte Proben trüb bleiben, bewirkt eine normale phagozytotische Aktivität eine Klärung des Belebtschlammüberstandes (Abbildung 1).

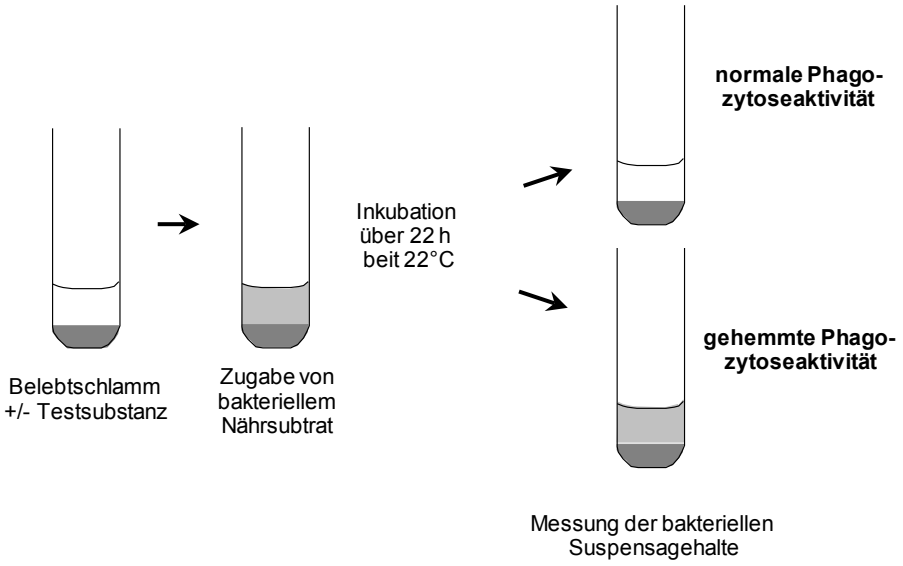

Abbildung 1: Testdesign zur Erfassung der Hemmwirkung von Chemikalien auf die Phaghozytoseleistung der Protozoenbiozönose in Belebtschlämmen.

Aufbauend auf den Ergebnissen dieser Testentwicklung wurde im Rahmen einer wiederum vom Umweltbundesamt geförderten Pilotstudie das Testprotokoll in den Jahren 2005und 2006 harmonisiert und weiter validiert (Pauli & Poka, 2007). Anhand dieses ersten Validierungsschrittes, in dem Belebtschlämme von vier unterschiedlichen Belebungsanlagen Deutschlands, Dänemarks und Österreichs einbezogen wurden, konnte gezeigt werden, dass bereits ein hohes Maß an Standardisierung des Tests erreicht ist und mit dem vorliegenden Testprotokoll reproduzierbare Ergebnisse erzielt werden können.

Zielstellung

Nach dieser Vorvalidierung sollte nun eine abschließende Validierung in Form einer statistisch belastbaren Ringstudie unter Berücksichtigung der OECD-Vorgaben des „Guidance Document on the validation and international acceptance of new or up-dated test methods for hazard assessment" erfolgen.

In den Jahren 2012 bis 2014 wurde dementsprechend ein Ringtest mit einem überarbeiteten Testprotokoll durchgeführt. Auftraggeber war das Umweltbundesamt. Die Koordination und Organisation übernahm die Freie Universität Berlin. Neben der Freien Universität Berlin nahmen vier GLP-zertifizierte Labors an der Ringstudie teil (vgl. Tabelle 1).

Mit Ausnahme des Labors an der FU Berlin hatte keines der teilnehmenden Labors Erfahrungen mit dem Protozoen-Belebtschlammtest. Allerdings lagen in 3 dieser Labore Kenntnisse zum Atmungshemmtest mit Belebtschlamm vor. Nur in einem Fall bestanden keinerlei Erfahrungen im Umgang mit Belebtschlamm als „Testorganismus".

Tabelle 1: Teilnehmer am Ringtest.

Labor	Land
CERI	Japan
Dr. Noack	Deutschland
Eurofins	Deutschland
Freie Universität Berlin	Deutschland
Umweltbundesamt	Deutschland

Die Validierung des Tests erfolgte anhand von fünf umweltrelevanten Chemikalien (Tabelle 2). Im Test wurden dabei jeweils drei Parallelansätze (Triplikate) für die Testsubstanzen mitgeführt werden. Mit den Ergebnissen sollte eine Basis für biometrische Analysen geschaffen werden, um Aussagen zur Interlaborvarianz des Tests zu ermöglichen.

Von der Freien Universität wurde ein Großteil des Testmaterials zur Verfügung gestellt. Die Teilnehmer erhielten zudem ausführliche Instruktionen zur Testdurchführung in Form von schriftlichen Anleitung und Videobei-

spielen. Zur Testdokumentation wurden von der Koordination speziell für den Test entworfene MS Excel Vorlagen bereitgestellt. Die Rohdaten aller Teilnehmer wurden schließlich an der FU Berlin gesammelt und statistisch ausgewertet. Die Ergebnisse dieser Ringstudie bilden die Grundlage für diesen Bericht.

Tabelle 2: Testsubstanzen der Ringstudie.

Chemikalie	Bezug	CAS Nr.	logPow	vermuteter Wirkmodus
1-Octylamin	Fluka	111-86-4	0.76 (1)	polar narcosis (2)
3,5-Dichlorophenol	Fluka	591-35-5	3.68 (3)	polar narcosis (4)
Dimethylsulfoxide	Sigma	67-68-5	-1.35 (5)	nonpolar narcosis (2)
Phenyl Ether	Fluka	101-84-8	4.21 (5)	nonpolar narcosis (6)
Hexachlorophen	Fluka	70-30-4	6.91 (7)	polar narcosis (8)

ref.: (1) Sigma-Aldrich (2) TETRATOX 2012 (3) Taylor, 1990 (4) Cronin, et al., 2002 (5) Hansch et al. 1995 (6) Pauli, 2004 (7) Scherrer & Donovan, 2009 (8) Lessigiarska, 2006

ERGEBNISSE

Datenmaterial

Im Ringtest wurden in fünf verschiedenen Labors Vergleichstests mit fünf Testsubstanzen durchgeführt. In allen Labors erfolgten Messungen mit mindestens fünf Konzentrationsabstufungen der jeweiligen Testsubstanz als 3-facher Parallelansatz (Triplikate). Für die unbehandelten (Negativ-) Kontrollen sowie die mit Digitonin defaunierten (Positiv-) Kontrollen wurden jeweils 6-fach Parallelansätze im Test mitgeführt. In einem Labor wurden einige Substanzen mehrfach, d.h. an verschiedenen Tagen und mit unterschiedlichen Belebtschlämmen, untersucht (vgl. Tabelle 3).

Tabelle 3: Übersicht zum Datenmaterial - Testsubstanzen und Anzahl der teilnehmenden Labors (großes X: mehrere Testläufe durchgeführt (Index = Anzahl Testläufe).

	Labore				
Chemikalien	**1**	**2**	**3**	**4**	**5**
1-Octylamin	x	x	x	x	X(2)
3,5-DCP	x	x	x	x	X(3)
DMSO	x	x	x	x	X(2)
Hexachlorophen	x	x	x	x	X(2)
Phenyl Ether	x	x	x	x	X(2)

Kontrollen

Ziel der Kontrollen ist es, die normale, unbeeinträchtigte Phagozytoseaktivität der Protozoen abzubilden. Erreicht wird dies, indem die Abnahme bakteriellen Substrats über einen knapp eintägigen Zeitraum verfolgt wird. Da das als „Futter" zugesetzte bakterielle Substrat eine deutliche Trübung hervorruft, lässt sich dieser Fraßvorgang photometrisch, d.h. nicht-invasiv, über die optische Dichte (OD; hier bei 440 nm) erfassen. Allerdings nimmt der Bakteriengehalt im Versuchsansatz nicht nur durch die Fraßleistung der Fauna ab, sondern ein kleiner Teil des bakteriellen Suspensagehaltes schwindet auch durch Aggregationsvorgänge und unspezifische Bindung der bakteriellen Partikel an die Belebtschlammflocken im Testansatz. Zusätzlich zu den eigentlichen, unbehandelten (Negativ-) Kontrollen erfordert dies das Mitführen weiterer, Phagozytose-freier Kontrollen, die anzeigen, welcher Anteil der Abnahme der Trübung (resp. optischen Dichte) auf unspezifische Veränderungen der Trübung während der Testphase zurückzuführen ist. Solche (Negativ-) Kontrollen werden erreicht, indem einem Teil der unbehandelten Kontrollen die chemische Substanz Digitonin zugegeben wird. Digitonin wirkt eukaryotenspezifisch und defauniert innerhalb weniger Minuten die Belebtschlammprobe. In der so mit Digitonin von jeglicher Fauna befreiten, defaunierten (def) Kontrolle findet keine Phagozytose mehr statt; alle jetzt noch messbaren Veränderungen der Trübung spiegeln unspezifische Veränderungen der Trübung - unbeeinflusst von Protozoen - wider. Die reine Phagozytoseaktivität ΔOD_{corr} lässt sich nun ermitteln, indem von der Abnahme der Trübung der unbehandelten Kontrollen ΔOD die Abnahme der Digitonin-Kontrollen ΔOD_{def} subtrahiert wird (vgl. folgende Gleichung). Die Gesamt-Abnahme, die mit der unbehandelten (Negativ-) Kontrolle erfasst wird, beinhaltet zusätzlich zum Verschwinden der Bakterien durch Phagozytose noch den unspezifischen Schwund (vgl. Abbildung 2):

$$\text{Phagozytoseaktivität } (\Delta OD_{corr}) = \text{Gesamttrübungsabnahme } (\Delta OD) - \text{unspezifischer Schwund } (\Delta OD_{def})$$

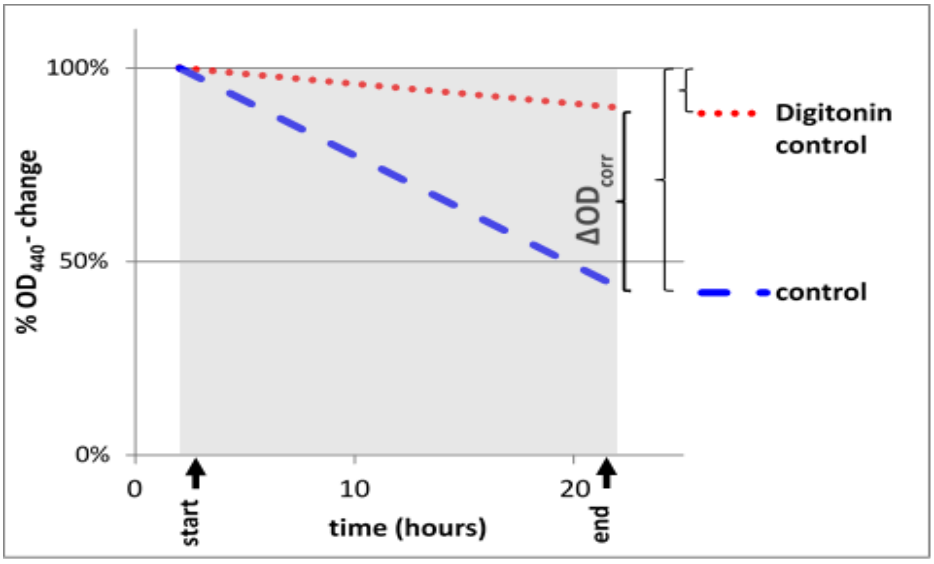

Abbildung 2: schematische Darstellung der OD-Abnahme der (unbehandelten) Kontrolle sowie der Digitonin-Kontrolle über den 22-stündigen Testzeitraum. Die Phagozytoseaktivität ergibt sich aus der Differenz der Gesamt-OD-Abnahme (ΔOD(control)) und der unspezifischen Abnahme, die mit der Digitonin-Kontrolle erfasst wird (ΔOD_{def}(control).

In der folgenden Tabelle (Tabelle 4) sind die Ergebnisse aller Kontrollläufe zusammengefasst. Insgesamt wurden 36 verschiedene Testläufe ausgewertet. In fünf Fällen wurde die im Testrichtlinienentwurf erforderliche Mindestphagozytoseaktivität von 25% (Validitätskriterium 1: $\Delta OD_{korr} > 25\%$[1]) im Belebtschlamm nicht erreicht. Diese fünf hinsichtlich der Phagozytoseaktivität nicht validen Fälle sind bei weiteren Berechnungen zur mittleren Fraß-aktivität nicht berücksichtigt. Die 36 aufgelisteten Testläufe reduzieren sich demnach auf 31 Messungen, die als Grundlage für die statistische Analyse der Phagozytoseaktivität her-angezogen wurden. Ausgehend von einem durchschnittlichen Wert von 1,35 nimmt die optische Dichte der Testansätze bei einer Wellenlänge von 440 nm im Testzeitraum um 0,74 OD-Einheiten (57%) im Mittel ab. Parallel hierzu zeigen die Messungen aller Teilnehmer eine unspezifische Abnahme, wie sie mit Hilfe der Digitonin-Kontrollen erfasst wird, um 0,13 OD-Einheiten (9%). Korrigiert um diesen letzteren Wert beträgt die allein auf die Fraßaktivität entfallende Trübungsabnahme im Mittel (meanΔODcorr) 0,62 OD-Einheiten bzw. 47%. Dies sind 22% mehr als es zur Erfüllung des Validitätskriteriums 1 (mean$\Delta OD_{corr} > 25\%$) nötig ist.

Tabelle 4: Zusammenfassung der optischen Dichtemessungen (OD-Werte) der unbehandelten (Negativ-) sowie der defaunierten (Digitonin = Positiv-) Kontrollen aller validen Testläufe (n=26).

	Kontrollen				
	2h_OD	22h_OD	ΔOD	ΔOD_{corr}	$\%\Delta OD_{corr}$
Mittel	1,35	0,61	0,74	0,62	47%
StAbw	0,13	0,26	0,18	0,17	15%
Median	1,36	0,66	0,74	0,61	44%
N	26	26	26	26	26
Min	0,96	0,11	0,43	0,37	26%
Max	1,57	1,01	1,19	1,03	79%
	defaunierte-Kontrollen				
	$2h\_OD_{def}$	$22h\_OD_{def}$		mittlereΔOD_{def}	$\%\Delta OD_{def}$
Mittel	1,45	1,33		0,13	9%
StAbw	0,07	0,08		0,03	2%
Median	1,46	1,34		0,12	8%
N	26	26		26	26
Min	1,34	1,20		0,06	4%
Max	1,61	1,47		0,20	14%

[1] Bei einer mittleren Start-OD von 1,35 (vgl. Tabelle 4 → 2h_OD) bedeutet das Validitätskriterium einer fraßbedingten ΔOD_{korr} in Höhe von mindestens 25% bezogen auf die Start-OD eine Mindestabnahme der Kontroll-Mittelwerte (ΔOD_{korr}) von 0,34 OD-Einheiten im 22-stündigen Messzeitraum. Andernfalls gilt der Test als ungültig bzw. die Auswertung als in hohem Maße fehleranfällig. Dies jedenfalls lässt sich empirisch aus Erfahrungen mit Belebtschlämmen besonders niedriger Aktivität herleiten. Um die Robustheit des Tests zu erhöhen, wird vom Validation Management sogar eine noch höhere Mindestabnahme von 30% (dies würde hier eine ΔODkorr > 0,41 bedeuten) vorgeschlagen, was bei einer mittleren ΔOD_{korr} von 0,62 aller Testteilnehmer (n=26) auch i.d.R. gewährleistet scheint (nur im Falle eines Labors waren 5 Tests nicht valide, was hier mit einer außergewöhnlich geringen Belebtschlammaktivität über einen mehrmonatigen Zeitraum zusammenhängt, die in dieser Höhe und in diesem Ausmaß nach den Erfahrungen des Validation Management der FU Berlin als Ausnahmesituation einzustufen ist, die bei entsprechender Erfahrung des Labors vermeidbar ist).

Eine hohe Phagozytoseaktivität hat im Vergleich zu einer geringen Aktivität einen großen Unterschied zwischen Start- und End-OD der Kontrollen nach 22 Stunden zur Folge. Auf Grund dieser größeren Spreitung lassen sich damit einhergehend auch Hemmeffekte durch Chemikalieneinwirkung deutlicher erkennen als es bei einer geringen Fraßaktivität der Fall wäre. In „normalen" Fällen nimmt die Trübung fraßbedingt 40-70% ab. Geringere Abnahmen führen zu zunehmend schlechter kalkulierbaren Chemikalieneffekten, da einerseits die OD-Unterschiede der Kontrollen immer geringer werden und andererseits die Unterschiede zwischen Kontrollen und Chemikalien-behandelten Testansätzen immer verschwommener abgebildet werden. Dies bestätigen jedenfalls Voruntersuchungen zur Festlegung von Test-Validitätskriterien. Der zu Beginn der Testphase des Ringtests hierbei festgelegte Minimalwert von 25% sollte auf 30% erhöht werden (→ mean ΔODcorr > 30%), um die Robustheit des Tests weiter zu erhöhen. Auf Basis der von den Teilnehmern eingereichten Messwerte scheint dies auch ohne Einschränkungen der allgemeinen Anwendbarkeit des Tests möglich: In nur einem Labor wurden geringere Phagozytoseaktivitäten beobachtet, die als Ausnahmesituation zu werten und durch ein Ausweichen auf Belebtschlämme anderer Klärwerke leicht vermeidbar sind.

Zur Bestimmung der Phagozytoseaktivität bzw. deren Hemmung durch Chemikalien ist neben einer genügend hohen Fraßaktivität auch eine möglichst geringe unspezifische Trübungsabnahme des bakteriellen Nährsubstrates von Bedeutung. Im Extremfall würde ein vollständiges Binden der als Futter zugesetzten Bakterien an den Belebtschlamm oder ein starkes Aggregieren von Einzelzellen zu großen bakteriellen Zellverbänden das Bestimmen des testentscheidenden Parameters Phagozytoseaktivität unmöglich machen. Das Substrat soll also möglichst wenig mit dem natürlichen System Belebtschlamm unspezifisch wechselwirken. Um dies kontrollieren zu können, werden in jedem Testlauf defaunierte (Digitonin-) Kontrollen mitgeführt, die die Stärke dieser Wechselwirkung anzeigen (vgl. Tabelle 2). Im Mittel (n=26) nimmt die anfängliche Trübung, gemessen als OD_{440}, um 0,13 OD-Einheiten ab. Dies entspricht einer mittleren Abnahme in allen Labors von 9%. Als Validitätskriterium 2 ist in dem den Untersuchungen zu Grunde liegenden Testguideline-Entwurf ein Wert von höchstens 25% genannt (ΔOD_{def} (Control) < 25%). Alle Testläufe erfüllen diese Vorgabe und sind demnach als valide einzustufen: In allen Testläufen liegt die Trübungsabnahme der Digitonin-Kontrollen aller validen Testläufe unterhalb von 15% (Tabelle 4).

CHEMIKALIENWIRKUNG

Curve fitting

Zur Auswertung standen 26 verschiedene Testläufe zur Verfügung. In allen fünf Labors wurden alle 5 Testchemikalien mindestens einmal gemessen und in allen Labors konnten für alle Testchemikalien Konzentrations-Wirkungskurven zur Berechnung von EC_{50}-Werten ermittelt werden. Auf Grund anfänglich sehr geringer Belebtschlammaktivitäten wurden in Labor 5 alle Chemikalienmessungen erneut zu einem späteren Zeitpunkt mit einem Schlamm höherer Aktivität wiederholt.

In 26 Fällen lagen – entsprechend den festgelegten Kriterien – valide Testergebnisse vor. Wie bereits erwähnt wurden 5 der 31 Testläufe auf Grund zu geringer Phagozytoseaktivität als nicht-valide klassifiziert. Von den insgesamt 26 verbliebenen Testläufen konnte in einem Fall keine Wirkung gemessen und damit kein EC_{50}-Wert ermittelt werden (ein Testlauf mit der flüchtigen Substanz Phenyl Ether).

Zur Ermittlung von Konzentrations-Wirkungskurven ist im Testguideline-Entwurf noch ein drittes Validitätskriterium genannt, das nicht die Aktivität der Kontrollen betrifft, sondern auf Wechselwirkungen besonders von hohen Chemikalienkonzentrationen mit dem Testsystem abzielt. Den Hintergrund bildet hier die Beobachtung, dass einige der Testchemikalien wie z.B. Octylamin, 3,5-Dichlorphenol oder auch Phenyl Ether in hohen Konzentrationen einen Anstieg der Trübung verursachen können[2], der den Nachweis der Phagozytoseaktivität und deren Hemmung mittels der optischen Dichte stark beeinträchtigt. Um dem Rechnung zu tragen, werden Messwerte von Chemikalienkonzentrationen, die in defaunierten Proben (Probe mit Chemikalie und Digitonin) zu einem mehr als 5%igen Anstieg der optischen Dichte (ΔOD_{def} (treatment) < -5%) im Versuchsverlauf führen, bei der statistischen Analyse der Konzentrations-Wirkungsbeziehung (curve fitting) und der daraus folgenden EC_{50}-Bestimmung nicht berücksichtigt. In vier Fällen wurde ein zu hoher Anstieg der Trübung infolge Chemikalieneinflusses gemessen (vgl. Anhang). Allerdings führte das Ausklammern dieser einzelnen Messwerte zu keiner Beeinträchtigung der Kurvenanpassung mittels nicht-linearer Regression und damit der EC_{50}-Berechnung.

Für den statistischen Vergleich der Inter- sowie Intralabordaten konnten so 25 EC_{50}-Werte aus validen Testläufen herangezogen werden (n=25). 24 der 25 EC_{50}-Werte wurden mittels nicht-linearer Anpassung einer symmetrischen, sigmoiden 4-Parameter „dose-response" Kurve berechnet. Nur in einem der 25 Testläufe konnte mit einer symmetrischen Kurvenform keine statistisch aussagekräftige Anpassung erreicht werten. Hier wurde eine asymmetrische Kurve (Richard's five-parameter dose-reponse curve) verwendet.

EC_{50}-Werte

Die EC_{50}-Werte wurden alle durch Anpassung nicht-linearer Kurven an normalisierte prozentuale Hemmeffekte in Abhängigkeit logarithmierter$_{10}$ Konzentrationen ermittelt. Mit einer Ausnahme konnten symmetrische sigmoide Kurven zufriedenstellend angepasst werden. In einem Fall konnte eine signifikante Anpassung nur durch Anpassung eines asymmetrischen Kurvenmodells erreicht werden.

Im Mittel – alle Substanzen berücksichtigt - weichen die EC_{50}-Werte zwischen den Labors um den Faktor 2,65-fach voneinander ab. Die Extremwerte liegen bei einer Abweichung um den Faktor 2,06 bis 3,45. Der mittlere Variationskoeffizient für die EC_{50}-Werte aller Messungen liegt bei 37% und variiert je nach Testsubstanz zwischen 28 und 50%.

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[2] nach bisherigen Erkenntnissen ist diese Trübungszunahme im Wesentlichen auf Veränderungen der Belebtschlammkonsistenz zurückzuführen. Da in defaunierten Proben unspezifische Veränderungen des Belebtschlammes direkt erfassbar sind, orientiert sich das dritte Validitätskriterium, das unerwünschte Wechselwirkungen von Chemikalien mit dem Testsystem auszuschließen versucht, an Digitonin-behandelten Proben.

Tabelle 5: Interlabor-Vergleich: Zusammenfassende Gegenüberstellung der EC50-Werte, wie sie zwischen (inter) den verschiedenen Labors ermittelt wurden.

	InterLabor-Vergleich			
	Verhältnis: max/min Wert	% Variations-koeffizient	Grubb's Grubbs's Test	n
1-Octylamin	3,10	48%	no outlier	5
35-DCP	3,45	50%	no outlier	5
DMSO	2,06	28%	no outlier	5
Hexachlorophen	2,20	29%	no outlier	5
Phenyl Ether	2,42	29%	no outlier	5
Mittelwert	2,65	37%		

DISKUSSION

Für die EC_{50}-Werte der verschiedenen Labors konnten mit Hilfe des Grubbs Tests keine signifikanten Ausreißer erkannt werden. Vom jeweiligen Mittelwert weichen dabei die EC_{50}-Werte der einzelnen Teilnehmer um das höchstens Zweifache ab. Die Variationskoeffizienten der EC_{50}-Werte zwischen den Labors streuen in einem Bereich von 30-50%.

Diese Höhe der Streuung ist als normal anzusehen und wird selbst bei hoch standardisierten Tests wie dem Leuchtbakterientest (30-55%%, (Nalecz-Jawecki, et al., 2010) oder dem Daphnientest (CVs überwiegen bis 40%, Rue, Fava, & Grothe, 1988) gefunden. Und selbst innerhalb eines Labors werden für den vergleichbaren Belebtschlamm-Respirationsinhibitionstest (OECD 209) Varianzkoeffizienten von 30-40% gemessen, Gendig et al., 2003).

Auf der Basis dieser Ergebnisse und unter Berücksichtigung der zwangsläufigen Unerfahrenheit von 4 der 5 Testteilnehmer ist der Protozoen-Belebtschlammtest als zuverlässig, anwenderfreundlich und bereits gut standardisiert anzusehen.

Keine Übereinstimmung der toxikologischen Daten zeigt sich beim Vergleich mit dem Belebtschlamm-Respirationsinhibitionstest (OECD Nr. 209). Hier scheint der Test mit Protozoen den bakteriellen Test und dessen ökotoxikologische Aussagen zu ergänzen und zu präzisieren statt zu ersetzten. Anders verhält es sich beim Vergleich mit EC-Werten von Ciliatentests. Sowohl Wachstumshemmtests als auch Phagozytosetests mit dem Ciliaten *Tetrahymena* ergeben vergleichbare Resultate der Chemikalieneffekte.

Summary

BACKGROUND

Protozoa and waste water treatment

The principle of biological sewage treatment plants is to transform the organic matter of incoming wastewater in microbial biomass, which in turn is separated from the liquid, yielding a purified effluent. The aim of this process is to achieve a maximal reduction of the organic load with a minimal bio-sludge production. The typical protozoan fauna of sewage treatment processes supports this process:

It is generally accepted that protozoan 'grazing' removes dispersed bacteria, resulting in higher transparency (clarification), i.e. lower suspended organic loads in the output water of the treated wastes (Pauli, et al., 2001).

Ecotoxicological relevance of protozoa

Ecotoxicological data show that in addition to bacterial tests, tests with activated sludge protozoa are indispensable to give a more accurate picture of the effects of pollutants towards the biological system of wastewater treatment (Pauli & Berger, 1999). It infers that these different functional groups supplement, rather than replace, each other with regard to the estimation of toxic effects in waste water treatment.

The status of protozoa as an important functional group, relevant for the risk assessment for sewage treatment plants has been acknowledged by the "Technical Recommendation" (ECB, 1998; TGD, 2003) of the European Economic Community. To assess chemical effects towards protozoa the "Technical Recommendation" points out a need for a testing strategy reflecting the integrity of the native ciliate population in sewage sludge as a whole.

Development of a protozoan test with activated sludge

In 2002, a R&D-project (Pauli & Poka, 2005) was initiated by the German Federal Environment Agency to meet the 'need(s) for testing data reflecting the integrity of the native ciliate population in sewage sludge as a whole (e.g. TGD, 1998, 2003; ECHA, 2014). The research project has been performed at the Free University of Berlin. The project aimed at developing an activated sludge test to assess the functional integrity of protozoa, the main consumers of suspended bacteria in activated sludge technology.

A test was developed that monitors the bacterivorous activity of (native) activated sludge[3] fed with bacteria (compare also the following Figure 3). The elimination of these added bacteria is measured by photometric means. The test mimics this natural removal of bacteria: Activated sludge is taken from the outlet of the aeration basin which contains already clarified wastewater. In the laboratory bacteria are added artificially to this effluent mixed liquor (instead of the bacteria from the incoming sewage) and the subsequent clearing is taken as a measure for the phagocytic activity. The test temperature of 22°C is close to natural conditions. The temperature of the

[3] Due to its importance and the ease of accessibility the investigations were focused on activated sludge from municipal treatment plants.

activated sludge, contrary to the assumption, varies mainly between 15 and 25°C [14] throughout the year in a large municipal sewage treatment plant. The reason for this relatively low variation compared to air temperatures is the constant temperature of the wastewater from individual households (e.g. washing machines and dishwashers). Under real conditions a drop of water takes ca. one day to pass through a modern activated sludge plant. In this time period the sewage circulates several times through the wastewater treatment plant. With an incubation period of 22hrs an attempt is also be made here to emulate the natural process.

To assess chemical effects the test substance is applied in various concentrations to activated sludge samples. The test is performed in mid-size glass test tubes with a culture volume of two mL and runs under static conditions over a time span of 22 hours. The tubes are closed with oxygen permeable caps and shaken at 22°C. The percent reduction of the activity in relation to an untreated control is calculated on the basis of the optical density readings. Effective concentrations (EC-values) are derived by statistical means.

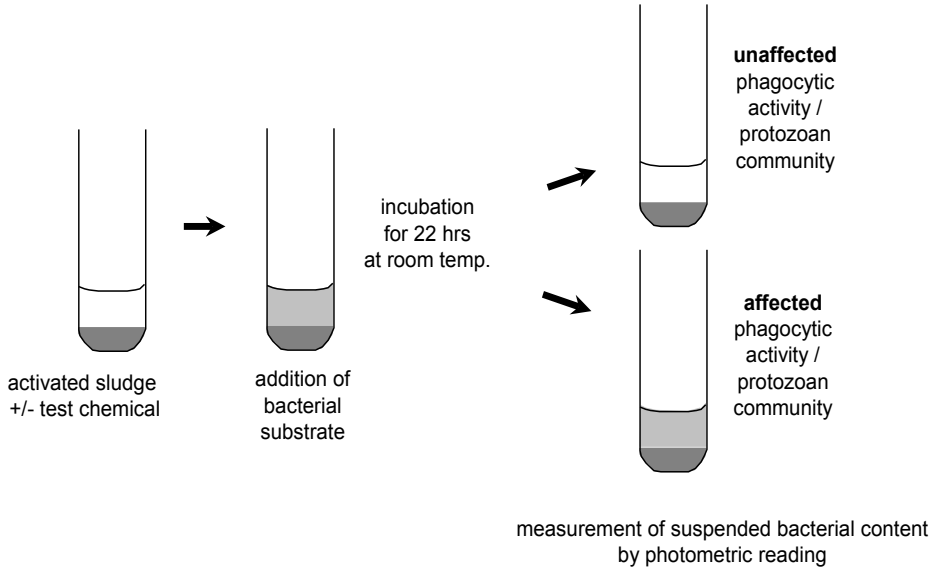

Figure 3: Scheme for the evaluation of chemical effects on the (phagocytic) function of the protozoan community in sewage sludge probes.

Pilot study

A round robin with financial support from the German Federal Environment Agency was organized in the years 2005 and 2006 to assess the standardization potential of the protozoan activated sludge test. Inter-calibrating exercises have been carried out on the basis of a first draft of a Test Guideline with three reference chemicals involving four labs using activated sludge from four municipal waste-water treatment plants in Denmark, Austria and Germany (Pauli & Poka, 2007).

Activated sludge is, by nature, a variable commodity, and varying results can occur from day to day as a result of shifts in the bacterial population. Therefore, in practice, EC_{50}-values from activated sludge tests are expected to vary considerably more than those from tests with well-defined test organisms.

Taking this into account, the results of the protozoan activated sludge tests indicate an already relatively high degree of standardization of the draft test protocol. The observed coefficients of variations (CVs) of around 40% correspond well to between-source variations of the OECD respiration inhibition test and even to between-laboratory %CVs found for fully standardized single species ecotoxicity tests.

Final ring study

After the pilot study to harmonize the test protocol the German Federal Environment Agency (UBA) decided to perform a final validation study (coordinated by the Free University of Berlin) involving international laboratories in order to evaluate the inter-laboratory reproducibility of the test.

Between 2012 and 2014 this final ring test took place with the objective to formally validate the 'protozoan activated sludge test' on the basis of a revised second version of the draft guideline. It was organized and coordinated by the Free University of Berlin on behalf of, and with funding from the German Federal Environment Agency. Tests were performed at the Free University of Berlin and at an additional four laboratories (see Table 6).

Table 6: Participating laboratories in the ring test (CERI: Chemicals Evaluation and Research Institute; Eurofins: Eurofins Agroscience Services EcoChem GmbH; UBA: German Federal Environment Agency).

Laboratory	Country
CERI	Japan
Dr. Noack	Germany
Eurofins	Germany
Free University of Berlin	Germany
German Environmental Agency	Germany

Except for the laboratory of the Free University of Berlin, none of the participants had previous experience with the protozoan test system. The inter-laboratory validation study consists of assays with three replicates (three parallels) using the following five model chemicals with (supposed) nonpolar and polar narcosis type of action

and spanning a wide range of physico-chemical properties (Table 7). All data from test runs were gathered at the Free University of Berlin and statistically analyzed to interpret the results and reproducibility of the analytical parameters calculated as EC_{50}. The results of that validation step are the subject of this report.

Table 7: Substances included in the final ring test.

Substance	source	CAS No.	logPow	susp. mode of action
1-octylamine	Fluka	111-86-4	0.76 (1)	polar narcosis (2)
3,5-dichlorophenol	Fluka	591-35-5	3.68 (3)	polar narcosis (4)
Dimethyl sulfoxide	Sigma	67-68-5	-1.35 (5)	nonpolar narcosis (2)
phenyl ether	Fluka	101-84-8	4.21 (5)	nonpolar narcosis (6)
hexachlorophene	Fluka	70-30-4	6.91 (7)	polar narcosis (8)

ref.: (1) Sigma-Aldrich (2) TETRATOX 2012 (3) Taylor, 1990 (4) Cronin, et al., 2002 (5) Hansch et al. 1995 (6) Pauli, 2004 (7) Scherrer & Donovan, 2009 (8) Lessigiarska, 2006

RESULTS

Data basis

Five laboratories participated in this collaborative validation study. In all labs tests were performed with five model chemicals using at least five concentrations steps (three replicates per step). Each test series included two controls: one control without chemical addition to verify chemical effects (negative control), and one control containing digitonin to totally inhibit phagocytic activity (positive control). Both controls were run with six parallels each.

In total, 31 test runs were performed (comp. Table 8). All participants obtained valid data sets for each test chemical. In four laboratories all test runs proved to be in compliance with the validity criteria, in one laboratory 5 out of 11 test runs failed to comply with the validity criteria. This was mainly attributed to an unusually low phagocytic activity of the respective activated sludge samples. EC_{50}-values could be calculated for 25 out of the remaining valid 26 test runs. In one case no chemical effect could be observed.

Table 8: number of valid and total (parentheses) test runs.

	laboratories				
chemicals	**1**	**2**	**3**	**4**	**5**
1-octylamine	1 (1)	1 (1)	1 (1)	1 (1)	1 (2)
3,5-DCP	1 (1)	1 (1)	1 (1)	1 (1)	1 (3)
DMSO	1 (1)	1 (1)	1 (1)	1 (1)	1 (2)
Hexachlorophene	1 (1)	1 (1)	1 (1)	1 (1)	1 (2)
phenyl ether	1 (1)	1 (1)	1 (1)	1 (1)	2 (2)

Controls

To calculate the reduction in turbidity, the difference between the initial optical density (OD-measurement 1) and the second measurement after the incubation period (OD-measurement 2) was taken: In the presence of protozoa a reduction in turbidity (caused by the addition of *E. coli*) can be observed (ΔOD); in the probes with the eukaryotic inhibitor digitonin, the difference between measurements reflects non-specific changes in turbidity, independent of phagocytic activity (=ΔOD_{def}). To correct for this unspecific change, the actual phagocytic activity (ΔOD_{corr}) of each activated sludge sample was calculated by subtracting the average value of the defaunated parallel treatments (mean ΔOD_{def}) from the total OD-decrease of each replicate (ΔOD), see also Figure 4:

$$\Delta ODcorr = \Delta OD - \text{mean } \Delta OD_{def} \qquad \text{Eq. 1}$$

In five cases out of 31 test runs the predetermined minimum level of 25% phagocytic activity in the activated sludge sample (ΔOD_{corr} > 25%, validity criterion 1[4]) was not satisfied. Data from these five runs were excluded from further statistical analysis of chemical effects. Consequently, the EC_{50} evaluation refers to 26 valid test runs. On average (all valid test runs from all participants included) the optical density at 440 nm (OD_{440}) starts with values of 1.35 (Table 9). This value decreases for 0.74 OD-units to values of 0.62. The part of this decrease that is due to unspecific, non-phagocytosis dependent alterations amounts to 0.13 OD-units. The OD-decrease of untreated controls is corrected by this value resulting in a mean phagocytosis activity (ΔOD) of 0.62 OD-units within 20 hrs. Thus, the percentage reduction of turbidity by engulfment of bacterial food amounts on average to 47%, which is almost the double of the predetermined minimum value of 25%.

[4] On average the phagocytosis dependent OD-decrease (% ΔOD) amounts to 47% (see). The minimum value of this decrease is set to 25%, otherwise the test run is considered invalid. According to the experience of the validation management this minimum is required to achieve reliable measurements.

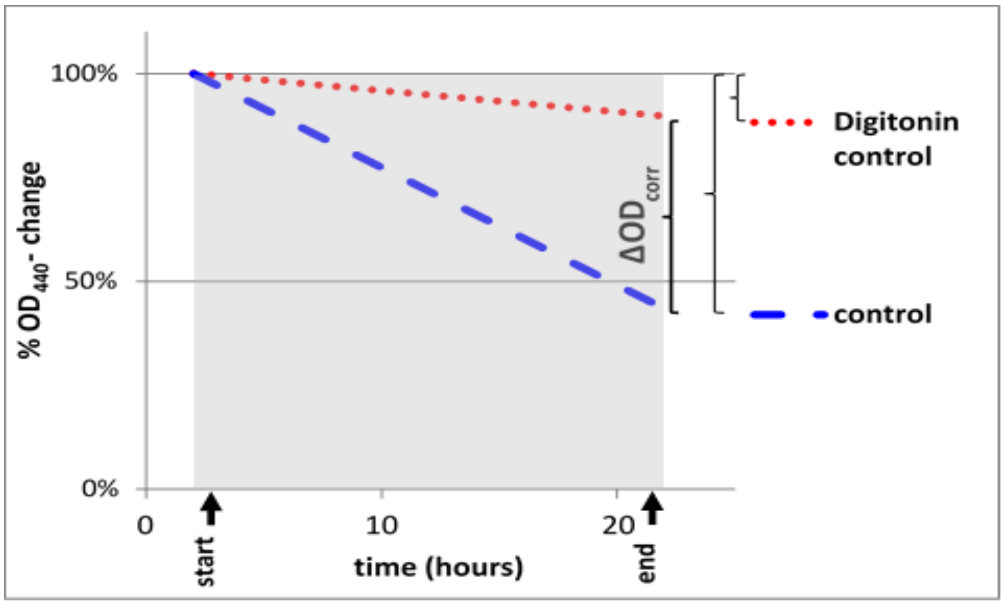

Figure 4: schematic diagram of the OD-decrease over the 20hr-test period in both untreated and defaunated (digitonin-treated) controls. To determine the phagocytic activity (∆ ODcorr) the total OD-decrease needs to be corrected by subtracting the phagocytosis-independent OD-change of defaunated, i.e. digitonin-treated controls.

Table 9: Summary of the valid test runs for both untreated and defaunated controls.

	controls				
	2h_OD	22h_OD	ΔOD	ΔOD_{corr}	$\%\Delta OD_{corr}$
mean	1,35	0,61	0,74	0,62	47%
StDev	0,13	0,26	0,18	0,17	15%
median	1,36	0,66	0,74	0,61	44%
n	26	26	26	26	26
min	0,96	0,11	0,43	0,37	26%
max	1,57	1,01	1,19	1,03	79%
	defaunated-controls				
	$2h\_OD_{def}$	$22h\_OD_{def}$		mean ΔOD_{def}	$\%\Delta OD_{def}$
mean	1,45	1,33		0,13	9%
StDev	0,07	0,08		0,03	2%
median	1,46	1,34		0,12	8%
n	26	26		26	26
min	1,34	1,20		0,06	4%
max	1,61	1,47		0,20	14%

Concentration-response relationships

In total the results of 26 valid test runs were available, five for each of the four test chemicals octylamine, 3,5-dichlorophenol, DMSO and hexachlorophene and six for the test substance phenyl ether.

In one valid test run with the (volatile) substance phenyl ether no effects could be observed over the whole concentration range. Consequently, no EC_{50}-value could be calculated. As a result, 25 EC_{50}-data served as the basis for the statistical evaluation of the ring test.

At high concentrations some chemicals tend to increase the turbidity of activated sludge. This may adversely affect the OD-measurement of the phagocytotic activity. This is the case for the highest concentration in four test runs with the test chemicals octylamine, 3,5-dichlorophenol and phenyl ether. Curve fitting and EC_{50}-calculation were performed omitting these data.

EC_{50}-values

For the statistical comparison concentration-response data from 25 test runs were available (five from each laboratory for each of the five test chemicals). Out of all recorded 31 test runs five had to be rejected as invalid (phagocytotic activity below the validity criterion) and in one case no effect could be measured. In four test runs the highest test concentrations had to be excluded from data analysis due to failing the requirements of validity criterion 3 (chemical induced OD-increase).

The EC_{50}-values were calculated by non-linear curve fitting to log-transformed concentrations and normalized %inhibition values. In all but one test run data were successfully fitted to a symmetrical sigmoidal curve. In case of one test run good fit results could only be obtained by applying an asymmetric function.

Within the test set the EC_{50}-values cover more than four orders of magnitude varying from 1.3 mg/L in the case of hexachlorophene and 3.1 g/L for dimethyl sulfoxide (mean values of all participants).

EC_{50}-data vary across laboratories on average by a factor of 2.60 with a mean coefficient of variation (CV) of 36%. The extreme values of the inter-lab EC_{50}-deviation are going from 2-fold (DMSO) to 3.45-fold (3,5-DCP) with coefficients of variation in the range of 27% to 49% (see Table 10).

Table 10: summarizes the inter-laboratory deviations of the EC_{50}-values for all test chemicals.

	between (inter-) lab comparison			
	ratio highest to lowest	Coefficient of variance	Grubb's test	n
1-Octylamine	3.10	48%	no outlier	5
35-DCP	3.45	49%	no outlier	5
DMSO	2.00	27%	no outlier	5
hexachlorophene	2.20	29%	no outlier	5
phenyl ether	2.26	29%	no outlier	5
mean	2.60	36%		

DISCUSSION

All EC_{50}-values were measured in sludge probes from different plants and in different labs. No valid data exist for intra-laboratory variability. Therefore no differentiated conclusions can be drawn with respect to intra- and exclusive inter-laboratory variability. However, the real situation of testing the phagocytic activity of native activated sludge implies not only tests in different labs but also continuously varying 'test organisms'. It might be reasonably assumed that in addition to inter-laboratory variability the differing sludge properties also play a dominant role in determining the variances of EC-values. Within-plant variations can occur from day to day as a result of shifts in the bacterial population. Sludge from different sources, and grown under different conditions, may also vary in response to inhibitors, because of varying degrees of reaction of some inhibitors with non-living sludge components. Therefore, in practice, EC_{50} values are also likely to be related to the specific activated sludge used for the phagocytosis test and inter- as well as intra-laboratory variations are only partly responsible for the scattering of effective concentrations.

Taking into account that four of the five participating laboratories had no previous experience with protozoa testing and that activated sludge tests are expected to vary considerably more than those from tests with well-defined test organisms the outcome of the ring test indicates an already relatively high degree of standardization of the test protocol: All laboratories obtained valid concentration response relationships for all model chemicals without previous training. Non-valid test runs can be mainly attributed to low phagocytic activity of the particular sludge batch. The observed inter-laboratory coefficients of variation of the EC_{50}-values of around 40% are within the normal range of fully standardized single species ecotoxicity tests.

For each of the 5 ring test chemicals, toxicological data for ciliate growth and phagocytosis were available. Data from the bacterial respiration inhibition test with activated sludge (OECD No. 209) were found for three of the test chemicals: The protozoan activated sludge test seems to have a different toxic profile as compared to the bacterial respiration inhibition test with activated sludge. It infers that these different functional groups supplement,

rather than replace, each other with regard to the estimation of toxic effects in waste water treatment. However, a high degree of correspondence is found with growth and phagocytosis tests of the ciliate *Tetrahymena*.

1 BACKGROUND

1.1 Protozoa and waste water treatment

The principle of biological sewage treatment plants is to transform the organic matter of incoming wastewater in microbial biomass, which in turn is separated from the liquid, yielding a purified effluent. The aim of this process is to achieve a maximal reduction of the organic load with a minimal bio-sludge production. The typical protozoan fauna of sewage treatment processes support this process:

It is generally accepted that protozoan 'grazing' removes dispersed bacteria, resulting in higher transparency (clarification), i.e. lower suspended organic loads in the output water of the treated wastes.

- Their predation upon bacteria also helps to 'sanitize' the water by diminishing pathogenic micro-organisms.
- There are strong indications that they increase the fraction of aggregated bacteria (flocs), thus facilitating gravitational separation of the sludge from the mixed liquor in activated sludge processes. The prey-predator relationship between protozoa and bacteria seems to be closely connected to this process of floc formation.
- Grazing by protozoa results in lower sludge production.
- Despite their antagonistic effect as predators of bacteria, protozoa are not considered detrimental to treatment efficiency. To the contrary, in the presence of protozoa, bacterial decomposition rates are often found to be stimulated, resulting in a lower dissolved organic content in the outflow.

The majority of microfaunal investigations confirms that all of the three main groups of protozoa - flagellates, ciliates and amoebae (naked and shell) - can be found in conventional wastewater treatment plants, whereby ciliates usually dominate over other protozoa not only in number of species but also in total count and biomass, both in activated sludge and in fixed-bed processes (percolation filters and rotating disc contactors).

1.2 Ecotoxicological relevance of protozoa

Ecotoxicological data show that in addition to bacterial tests, tests with activated sludge protozoa are indispensable to give a more accurate picture of the effects of pollutants towards the biological system of wastewater treatment (Pauli & Berger, 1999). It infers that these different functional groups supplement, rather than replace, each other with regard to the estimation of toxic effects in waste water treatment.

The status of protozoa as an important functional group, relevant for the risk assessment for sewage treatment plants, has been officially acknowledged (e.g. TGD, 1998, 2003; ECHA, 2014). To assess chemical effects towards protozoa these documents point out a need for a testing strategy reflecting the integrity of the native ciliate population in sewage sludge as a whole.

1.3 Development of a protozoan test with activated sludge

In 2002, a R&D-project (Pauli & Poka, 2005) was initiated by the German Federal Environment Agency to meet the 'need(s) for testing data reflecting the integrity of the native ciliate population in sewage sludge as a whole' (ECB, 1998; TGD, 2003). The research project has been performed at the Free University of Berlin. The project aimed at developing an activated sludge test to assess the functional integrity of protozoa, the main consumers of suspended bacteria in activated sludge technology.

Protozoa are responsible for clearing wastewater. They filter the particles out of the incoming water and prevent the formation of solitary bacteria suspensions in the treatment process. The test design should imitate this clearing performance under conditions that were as similar as possible to natural conditions.

A test was developed that monitors the bacterivorous activity of (native) activated sludge[5] fed with bacteria (compare also the following Figure 5). The elimination of these added bacteria is measured by photometric means. The test mimics this natural removal of bacteria: Activated sludge is taken from the outlet of the aeration basin which contains already clarified wastewater. In the laboratory bacteria are added artificially to this effluent mixed liquor (instead of the bacteria from the incoming sewage) and the subsequent clearing is taken as a measure for the phagocytic activity. The test temperature of 22°C is close to natural conditions. The temperature of the activated sludge, contrary to assumption, varies mainly between 15 and 25°C (Crites & Tchobanoglous, 1998) throughout the year in a large municipal sewage treatment plant. The reason for this relatively low variation compared to air temperatures is the constant temperature of the wastewater from individual households (e.g. washing machines and dishwashers). Under real conditions a drop of water takes ca. one day to pass through a modern activated sludge plant. In this time period the sewage circulates several times through the wastewater plant. With an incubation period of 22 hours an attempt is also be made here to emulate the natural process.

To assess chemical effects the test substance is applied in various concentrations to activated sludge samples. The test is performed in mid-size glass test tubes with a culture volume of two mL and runs under static conditions over a time span of 22 hours. The tubes are closed with oxygen permeable caps and shaken at room temperature (20-22°C). The percent reduction of the activity in relation to an untreated control is calculated on the basis of the optical density readings. Effective concentrations (EC-values) are derived by statistical means.

[5] Due to its importance and the ease of accessibility the investigations were focused on activated sludge from municipal treatment plants.

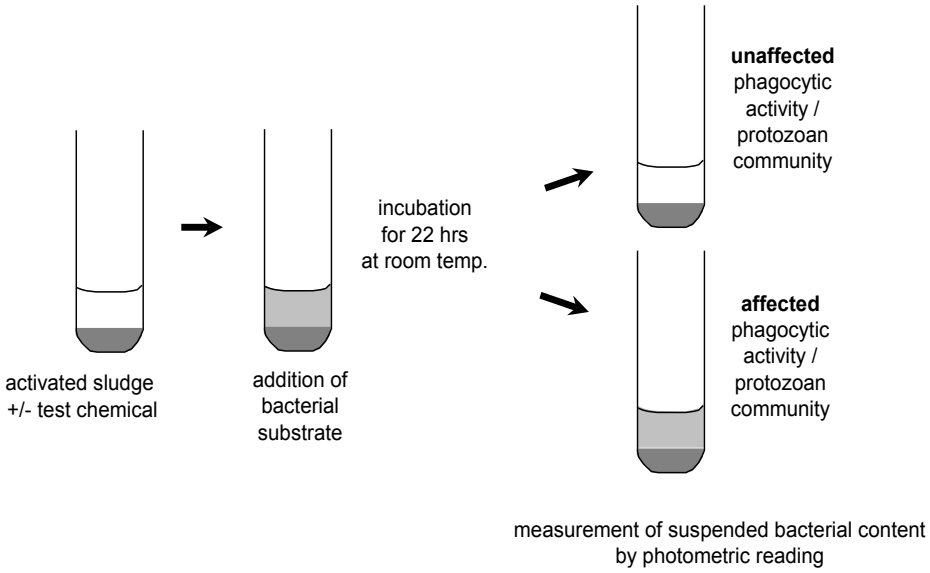

Figure 5: Scheme for the evaluation of chemical effects on the (phagocytic) function of the protozoan community in sewage sludge probes.

1.4 Pilot study

A round robin with financial support from the German Federal Environment Agency was organized in the years 2005 and 2006 to assess the standardization potential of the protozoan activated sludge test. Inter-calibrating exercises have been carried out on the basis of a first draft of a Test Guideline with three reference chemicals involving four labs using activated sludge from four municipal waste-water treatment plants in Denmark, Austria and Germany (Pauli & Poka, 2007).

Activated sludge is, by nature, a variable commodity, and varying results are often reported. Within-plant variations can occur from day to day as a result of shifts in the bacterial population. Sludge from different sources, and/or grown under different conditions, may also vary in response to inhibitors, because of varying degrees of reaction of some inhibitors with abiotic sludge components. Therefore, in practice, EC50-values from activated sludge tests are expected to vary considerably more than those from tests with well-defined test organisms.

Taking this into account, the results of the protozoan activated sludge tests indicated an already relatively high degree of standardization of the draft test protocol. The observed coefficients of variations (CVs) of around 40% corresponded well to between source variations of the OECD respiration inhibition test and even to between-laboratory %CVs found for fully standardized single species ecotoxicity tests.

1.5 Final ring study

After the pilot study to harmonize the test protocol the German Federal Environment Agency (UBA) decided to perform a final validation (coordinated by the Free University of Berlin) involving international laboratories in order to evaluate the inter-laboratory reproducibility of the test.

Between 2012 and 2014 this final ring test took place with the objective to formally validate the 'protozoan activated sludge test' on the basis of a revised second version of the draft guideline. It was organized and coordinated by the Free University of Berlin on behalf of, and with funding from the German Federal Environment Agency. Tests were performed at the Free University of Berlin and at an additional four laboratories. They are all GLP-certified and selected by the validation management at the Free University of Berlin at the suggestion of the UBA: one public institution and three contract laboratories (see Table 11).

Table 11: Participating laboratories in the ring test (CERI: Chemicals Evaluation and Research Institute; Eurofins: Eurofins Agroscience Services EcoChem GmbH; UBA: German Federal Environment Agency).

Laboratory	Country
CERI	Japan
Dr. Noack	Germany
Eurofins	Germany
Free University of Berlin	Germany
German Environmental Agency	Germany

Except for the laboratory of the Free University of Berlin, none of the participants had previous experience with the protozoan test system. However, four laboratories were familiar with activated sludge, i.e. with bacterial tests using activated sludge. Only one participating laboratory had no previous experience, neither with protozoa nor with bacterial activated sludge tests.

The inter-laboratory validation study consists of assays with three replicates (three parallels) using the following five model chemicals with (supposed) nonpolar and polar narcosis type of action and spanning a wide range of physico-chemical properties(Table 12). All data from test runs were gathered at the Free University of Berlin and statistically analyzed to interpret the results and reproducibility of the analytical parameters calculated as EC50. The results of that validation step are the subject of this report.

Table 12: Substances included in the final ring test.

Substance	source	CAS No.	logPow	susp. mode of action
1-octylamine	Fluka	111-86-4	0.76 (1)	polar narcosis (2)
3,5-dichlorophenol	Fluka	591-35-5	3.68 (3)	polar narcosis (4)
Dimethyl sulfoxide	Sigma	67-68-5	-1.35 (5)	nonpolar narcosis (2)
phenyl ether	Fluka	101-84-8	4.21 (5)	nonpolar narcosis (6)
hexachlorophene	Fluka	70-30-4	6.91 (7)	polar narcosis (8)

ref.: (1) Sigma-Aldrich (2) TETRATOX 2012 (3) Taylor, 1990 (4) Cronin, et al., 2002 (5) Hansch et al. 1995 (6) Pauli, 2004 (7) Scherrer & Donovan, 2009 (8) Lessigiarska, 2006

2 MATERIAL & METHODS

2.1 Material, equipment and instructions for participants

The coordinating laboratory at the Free University of Berlin provided most of the material and equipment to perform the test. Microsoft Excel templates were distributed to the laboratories for the test documentation and the collection of raw data. Additionally detailed testing instructions in text form and as video tutorials were made accessible to all participants via the Internet on the ring test website 'www. round-robin.info' (the written documentation is included in the Appendix II – Instructions to participants, starting on page 133). The following information and material were made available to each participant:

laboratory consumables:

substrate (lyophilized *E.coli*, ATCC 9637, Sigma-Aldrich)
dilution medium: EPA moderately hard (Microbiotests Inc., Belgium) pH-buffered with 10 mM HEPES (Serva), a detailed description of the preparation is APPENDIX VI - Test medium, page 157)
test chemicals as pure substances and partly in the form of stock solutions, respectively (see APPENDIX II – Instructions to participants, page 133)
digitonin solution (defaunating agent and positive control, Sigma-Aldrich)
test vials (screw top clear vial, 4 mL, Sigma-Supelco) with oxygen permeable screw caps (13 mm Black Top Hat Cap, PTFE/Silicone septum, Sigma-Supelco)

devices:

slant rack (40°) for 72 test vials (university's workshop),
if needed, a rotary shaker (Rotamax 120, Heidolph) and a small digital photometer (Libra S2, Biochrom) for measuring the samples in the test vessels (round glass vials) was provided

instructions - documents/SOP (see APPENDIX II – Instructions to participants):

Instructions for the preparation of media and chemical dilution steps
Standard Operating Procedure (SOP),
Bench protocol (Microsoft EXCEL workbook): a quick test preparation and documentation guide, containing the following three EXCEL sheets:

- Preparation sheet (EXCEL sheet1) + Preparation scheme (EXCEL sheet2)
- Reporting/Documentation sheet (EXCEL sheet3)

(lists, schemes and tables in the three EXCEL sheets were related to each other and communicate with each other, i.e. changes in the preparation sheet automatically updated the 2 other sheets),

instructions - videos:

Video1 -> sampling
Video 2 -> Aliquoting and pipetting the sludge
Video 2a -> cutting off the pipette tip
Video 2b -> sludge pipetting
Video 3 -> Digitonin dissolution
Video 4 -> dissolve *E. coli*
Video 5 -> incubation of test vials
Video 6 -> mixing - settling – optical density (OD) reading
Video 6a -> mixing of test vials & sludge settling
Video 6b -> OD measurement

2.2 Test substances

Five test substances were used in the ring test, namely 1-Octylamine (CAS No: 111-86-4), 3,5-dichlorophenol (CAS No: 591-35-5), Dimethyl sulfoxide (CAS No: 67-68-5), phenyl ether (CAS No: 101-84-8) and hexachlorophene (CAS No: 70-30-4). The chemicals were chosen because they cover a wide range of physicochemical properties with different (supposed) effective strength (compare 'test chemicals: description and preparation', page 96). In addition to the above reasons 3,5-dichlorophenol was included due to its general status as a reference standard in aquatic toxicity testing. Dimethyl sulfoxide was chosen because it is the preferred solvent in protozoan tests. It is a water miscible organic solvent used as a good solvent for poorly soluble organic test chemicals, showing a low toxicity towards protozoa. The volatile and therefore problematic substance phenyl ether was selected to verify the robustness and range of application of the test.

Four participating laboratories received the test chemicals from the Free University of Berlin. All these laboratories received chemicals from the same batch (see para 8, page 133: APPENDIX II – Instructions to participants). One laboratory (CERI, Japan) purchased all test chemicals from own suppliers.

2.3 Standard dilution water

As standard dilution water synthetic EPA-water (moderately hard), pH-buffered (10mM HEPES, pH 7.5) was chosen. Preliminary investigations revealed that this medium supports the phagocytic activity and minimizes unspecific binding reactions of suspended bacteria.

Detailed instructions on how to prepare the standard dilution water are given under Appendix II – Instructions to participants / Preparation of standard dilution medium, page 95).

2.4 Testing poorly water soluble substances

In case of the highly lipophilic (logPow 7.5) substance hexachlorophene, stock solutions were prepared in DMSO. DMSO was used in concentrations that did not exceed 0.2% (v/v), up to which no significant effects on phagocytic activity can be observed (own measurements).

2.5 'Test organism'

2.5.1 Sampling and storage

The activated sludge was taken with a scoop directly from the run off from the aeration tank of a municipal wastewater treatment plant (the powerful circulation in the aeration tanks ensures a homogeneity of the samples). At each sampling, ca. 0.5 – 1 L activated sludge was transferred into a screw-capped polypropylene container and transported to the laboratory. In the laboratory the necessary portion for experimental purposes was withdrawn from the polypropylene container. The remaining native activated sludge was stored in the screw-capped, transparent polypropylene container at 4-6°C for no longer than 5 days.

2.5.2 Dry substance

In most cases actual dry weight data (g/L) of the sampled activated sludge is provided by the wastewater laboratories which continuously perform routine measurements of the plants. If actual operating data was not available, the dry weight was determined by standard methods to measure total suspended solids concentration (TSS).

2.6 Substrate

All laboratories received the substrate from the Free University of Berlin. The substrate consisted of lyophilized bacteria[6], E. coli strain ATCC 9637 (risk group 1[7] test strain), being purchased from Sigma-Aldrich.

2.7 Sample preparation

For experimental purposes, a dilution series was prepared directly in the test vials by serial dilution of the highest test concentration in dilution medium. At least five test concentrations were used, following the range proposed in the instructions. The lowest and highest concentration were selected here to have no observed effect and to inhibit phagocytosis by more than 50% and preferably stopping phagotrophy altogether. Each dilution series was prepared in triplicate: One triplicate of the test series was run to verify chemical effects, while the other duplicate

[6] The freeze-dried bacterial substrate can be stored at -20°C for several months without any significant effects.

[7] Risk Group 1 (RG1): Agents that are not associated with disease in healthy adult humans.

included digitonin[8] (200 mg/L final concentration). This provided parallels with total inhibition of the phagocytic activity allowing the measurement of any passive reaction of the added *E. coli*-suspension, i.e. the nutrient substrate.

Each test run included six untreated controls in standard dilution water, which have been inoculated with activated sludge but without test chemicals being added (negative controls) and six controls containing activated sludge and digitonin to defaunate the samples (positive controls).

Consequently, each test run consisted of 6 controls plus at least 5 chemical treatment groups, each consisting of triplicate samples and the same quantity for defaunated, digitonin treated parallels (see Table 13).

To inoculate the test vials, the necessary volume of activated sludge was taken from the polypropylene container to give a final concentration of 1 g dry weight/L in the 2 mL assays[9]. Tests were started by the addition of a 50-fold concentrated *E.coli*-suspension (lyophilized bacteria reconstituted with H_2O, concentration in test: 0.4 mg/mL, which corresponds to an optical density of 1.3 - 1.4 at λ = 440 nm, path length ca. 13 mm).

Table 13: illustrates the number of test vials and treatment groups (control, conc. 1, conc. 2, …) for a typical test run of the protozoan activated sludge test.

	controls	chemical conc. 1	chemical conc. 2	chemical conc. 3	chemical conc. 4	chemical conc. 5
-	6 vials	3 vials	3 vials	3 vials	3 vials	3 vials
defaunated (digitonin-treated)*	6 vials	3 vials	3 vials	3 vials	3 vials	3 vials
Total	12	6	6	6	6	6

**Note: To correct for passive reactions of the substrate suspension (e.g. due to binding of the added E.coli to sludge flocs), the averaged values of the respective defaunated treatment* group are used for further calculations (see text below, para. 2.9).

2.8 Test procedure

Prior to the first OD-measurement the probes were equilibrated for 2 hours under vigorous shaking: all test vials were put in a holding tray and placed on a shaker at 250 rpm at 22±1°C (Note: during the incubation on the shaker it is important to keep the test vials at an angle of 20-40° to the horizontal. This facilitates optimal oxygen supply and lowers the aggregation/binding of the suspended bacterial food).

[8] The detergent digitonin selectively renders the eukaryotic plasma membrane permeable but not bacterial cells.

[9] The diluted activated sludge was transferred to the glass test tubes with an automatic pipette. To eliminate any floc-destroying shear forces, the pipette tip was cut off about 5 mm from the end, enlarging the tip aperture to ca. 3 mm.

After two hours of equilibration all samples were taken from the shaker, mixed thoroughly (vortexed) and left standing for 30 min. After sedimentation of the activated sludge, the supernatant was carefully (i.e. without disturbing the settled sludge[10]) measured in the 4 mL glass test tubes in a photometer with a cuvette slot for 15 mm round tubes at 440 nm (OD-measurement 1). Following the first photometric measurement of the supernatant, incubation on the shaker (250 rpm) continued for another 20 hrs at 22±1°C. After the incubation, the second OD-measurement of the turbidity of the supernatant (OD-measurement 2) was performed following the same procedure as the first.

2.9 Analyses

To calculate the reduction in turbidity, the difference between the initial optical density (OD-measurement 1) and the second measurement after the incubation period (OD-measurement 2) was taken: In the presence of protozoa a reduction in turbidity (caused by the addition of *E. coli*) can be observed (ΔOD); in the probes with the eukaryotic inhibitor digitonin, the difference between measurements reflects non-specific changes in turbidity, independent of phagocytic activity (=ΔOD_{def}). To correct for this unspecific change, the actual phagocytic activity (ΔOD_{corr}) of each activated sludge sample was calculated by subtracting the average value of the defaunated parallel treatments (mean ΔOD_{def}) from the total OD-decrease of each replicate (ΔOD):

$$\Delta OD_{corr} = \Delta OD - \text{mean } \Delta OD_{def} \qquad \text{Eq. 2}$$

Using this method, the phagocytotic activity (ΔODcorr) was calculated for each non-digitonin treated sample (6 control replicates and 3 replicates per chemical concentration). Based on the mean ΔODcorr of controls (n = 6) the inhibition for each replicate of the respective chemical treatment group was then calculated as a percentage to the averaged control:

$$\%\text{Inhibition}_x = 1 - \frac{\Delta OD_{corr}(\text{replicate}_x)}{\text{mean}\Delta OD_{corr}(\text{controls})} \times 100 \qquad \text{Eq. 3}$$

EC_{50}-values were determined statistically on the basis of these normalized values. The triplicate values of each chemical treatment group were treated as separate points. Concentration-response-curves were calculated by non-linear curve fitting to log-transformed concentrations and the percent inhibition values (Note: The data were normalized to the mean of control values. However, since there is random variation in the data, some data points have normalized values less than zero and others have normalized percentage values greater than hundred. They were left in to avoid biasing the analysis).

The top and bottom plateau were constrained to 0% and 100%, respectively. In all but one case a best fit could be achieved by the standard symmetrical model of a S-shaped 'four parameter dose-response' curve or 'Hill equa-

[10] The activated sludge must remain at the bottom of the test tube and the photometer must be built so that the beam of light passes through the supernatant above the sedimented sludge.

tion' (24 out of 25 concentration response curves). Two parameters (top and bottom) were constrained, so that only two parameters were fitted (the $logEC_{50}$ and the Hill Slope), compare the following Eq. 4:

$$y = \text{bottom} + \frac{top - bottom}{1 + 10^{(logEC50 - x) * HillSlope}};$$

x = log of chemical concentration, y = response;
y starts at Bottom and goes to Top with a sigmoid shape.

Eq. 4

In one case (one test run of laboratory 5 with 3,5-DCP) an adequate fit could only be obtained by fitting the common asymmetric model, known as the 'five parameter logistic equation' or the 'Richards function', shown here as Eq. 5:

$$y = \text{bottom} + \frac{top - bottom}{1 + 10^{((logXb - x) * HillSlope)^S}}$$

x = log of chemical concentration, y = response;
y starts at Bottom and goes to Top with a sigmoid shape;

$$logXb = logEC50 + {}^{1}/_{HillSlope} * \log(2^{\left(\frac{1}{S}\right)} - 1)$$

Eq. 5

Curve-fitting and the estimation of the EC_{50}-values and their 95% confidence limits was performed on the basis of non-linear regression using GraphPad Prism 6, GraphPad Software, San Diego California USA. To look for outliers in the recorded EC50-values that are either much higher than the other values or much lower, a two-sided Grubbs test (alpha=0.05) was run using the same statistics program.

Unless otherwise specified, all other statistical calculations were carried out using MS EXCEL standard formulas.

2.10 Validity criteria

Generally controls are included in a test run to show the normal, unimpaired activity. Here, this is achieved by monitoring the decrease of bacterial substrate of chemically untreated activated sludge probes for a period of nearly one day. Since the bacterial substrate causes a marked turbidity, this feeding process can be easily followed by non-invasive photometric measurements (OD-readings at 440 nm). However, the bacterial substrate in the sample decreases not only by the faunal feeding activity. Some part of bacteria decrease by self-aggregation and unspecific binding to the sludge flocs in the test sample. It is therefore essential to include a phagocytosis-free (positive) control, indicating what proportion of the decrease in turbidity (resp. optical density) is due to these nonspecific changes in the bacterial content during the test period. A selective inhibition of the phagocytosis can be achieved by the addition of the digitonin. This chemical selectively renders the eukaryotic plasma membrane

permeable but not bacterial cells. By the addition of digitonin the activated sludge sample can be defaunated within a few minutes and phagocytosis is stopped. Any changes in turbidity can now be attributed to passive reactions of the added bacteria - unaffected by phagotrophy. Subtracting this decrease in turbidity of digitonin controls from the decrease of turbidity of untreated controls yields the pure biological, i.e. phagocytic activity (comp. Figure 6).

A high phagocytic activity corresponds to a significant difference between the start and the final OD of controls. This also leads to a clearer distinction of chemical effects. On the other hand, low phagocytosis activity differences to controls tend to blur and chemical effect calculation becomes more and more imprecise.

In order to measure phagocytosis satisfactorily, the bacterial food should not only be taken up effectively but also show low adsorption. Unspecific interactions of the bacterial food should therefore be as minimal as possible. To check this, each test run includes defaunated, digitonin-treated controls with a total inhibition of the phagocytotic activity.

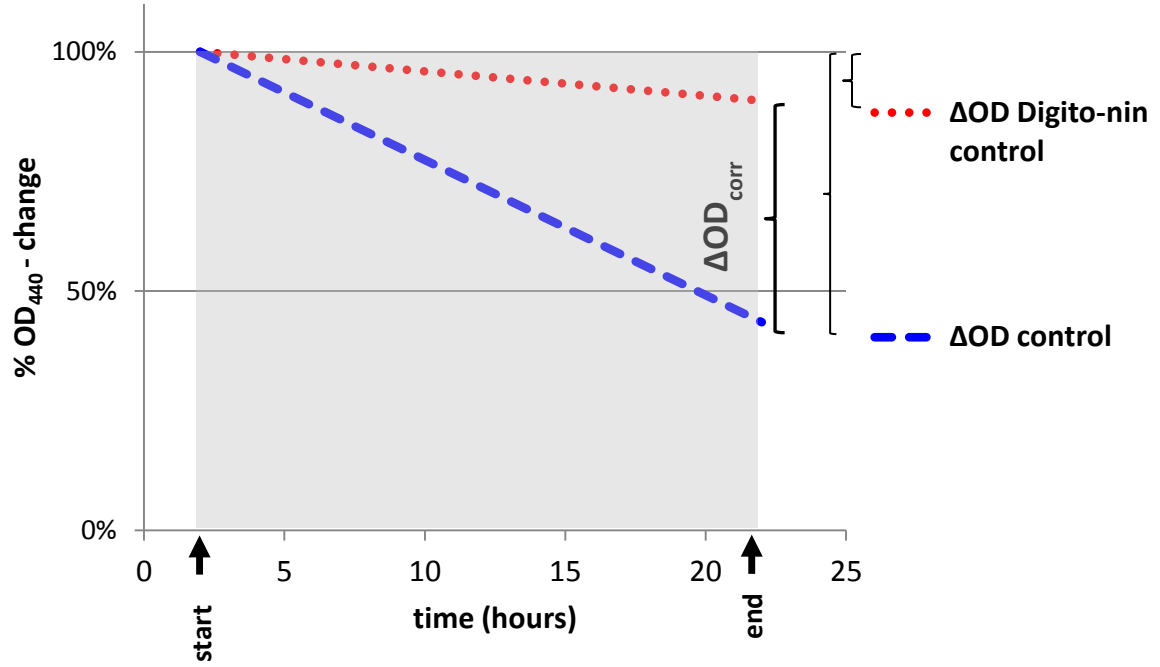

Figure 6: schematic diagram of the OD-decrease over the 20hr-test period in both untreated and defaunated (digitonin-treated) controls. To determine the phagocytic activity (Δ ODcorr) the total OD-decrease needs to be corrected by subtracting the phagocytosis-independent OD-change of defaunated, i.e. digitonin-treated controls.

At high concentrations some chemicals tend to increase the turbidity of activated sludge. This may adversely affect the OD-measurement of the phagocytotic activity. This must be taken into account when evaluating the data.

Consequently, based on the results of the pilot study (Pauli & Poka, 2007) and on preliminary investigations, the following three test validity criteria were included in the test protocol and used to select and reject data (Figure 7):

i) the mean phagocytotic activity (% ΔOD_{corr}(controls)) must exceed 25% within the testing time between 2 hrs and 22 hrs

ii) the mean of the unspecific OD-decrease (440 nm) of defaunated controls (%ΔOD_{def}(controls)) containing bacterial substrate and digitonin must remain below 25% within the testing time

iii) furthermore, the chemical test concentration at which a mean OD-increase (440 nm) of more than 5% in the defaunated parallel samples (containing bacterial substrate, digitonin and test chemical: %ΔOD_{def}(treatment)) occurs within the test interval has to be excluded from the effect calculation.

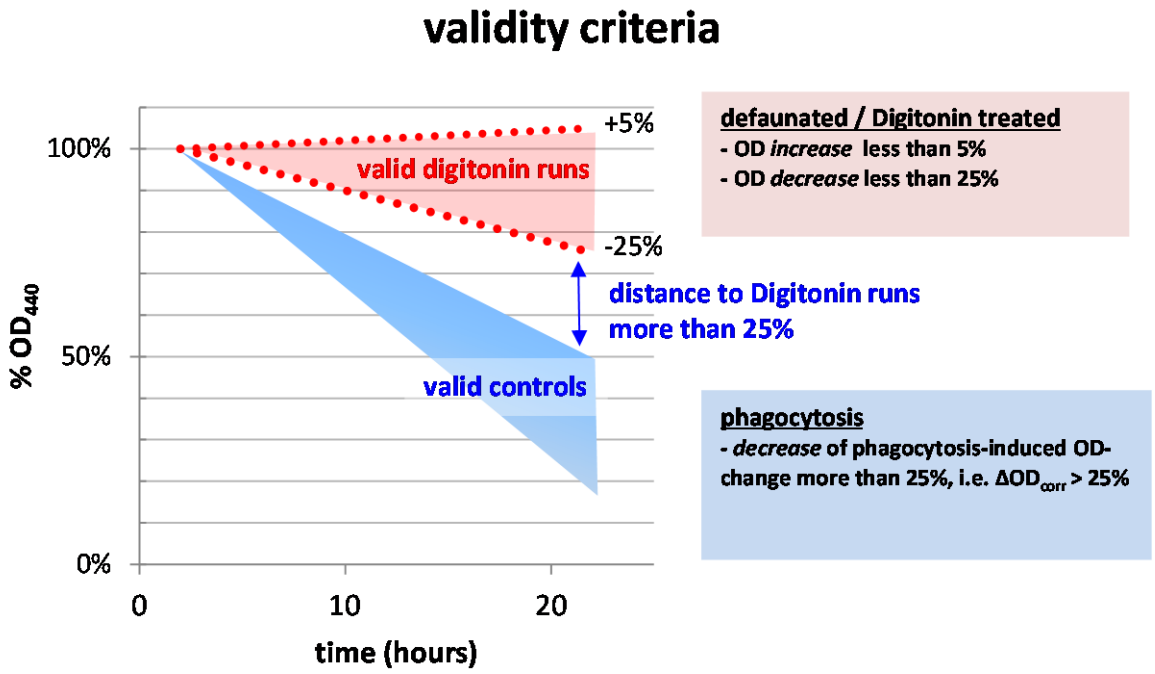

Figure 7: summarizes the validity criteria of the draft Guideline, which served as the basis for the ring study. Criterion 1: The phagocytosis activity – calculated as difference between the total OD-decrease and the OD decrease of defaunated controls – should be at least 25% within the test period. Criterion 2: The OD-decrease of defaunated (digitonin treated) controls must not exceed 25% in the test interval. Criterion 3: The OD-increase of test chemical-treated defaunated parallels should not be more than 5%.

3 RESULTS

3.1 Data basis

Five laboratories participated in this collaborative validation study. In all labs tests were performed with five model chemicals using at least five concentrations steps (three replicates per step). Each test series included two controls: one control without chemical addition to verify chemical effects (negative control), and one control containing digitonin to totally inhibit phagocytic activity (positive control). Both controls were run with six parallels each.

In total, 31 test runs were performed (comp. Table 14). All participants obtained valid data sets for each test chemical. In four laboratories all test runs proved to be in compliance with the validity criteria, in one laboratory 5 out of 11 test runs failed to comply with the validity criteria. This was mainly attributed to an unusually low phagocytic activity of the respective activated sludge samples. EC_{50}-values could be calculated for 25 out of the remaining valid 26 test runs. In one case no chemical effect could be observed.

Table 14: number of total and valid (parentheses) test runs.

	laboratories				
chemicals	**1**	**2**	**3**	**4**	**5**
1-octylamine	1 (1)	1 (1)	1 (1)	1 (1)	1 (2)
3,5-DCP	1 (1)	1 (1)	1 (1)	1 (1)	1 (3)
DMSO	1 (1)	1 (1)	1 (1)	1 (1)	1 (2)
Hexachlorophene	1 (1)	1 (1)	1 (1)	1 (1)	1 (2)
phenyl ether	1 (1)	1 (1)	1 (1)	1 (1)	2 (2)

3.2 Controls

The following Table 15 shows the recorded phagocytotic activity for all test runs. In five cases out of 31 test runs the predetermined minimum level of 25% phagocytic activity in the activated sludge sample (validity criterion 1[11]) was not fulfilled (comp. also Figure 7). Data from these 5 runs were excluded from further statistical analysis of chemical effects. Consequently, the EC_{50} evaluation refers to 26 valid test runs.

On average (all valid test runs from all participants included) the optical density at 440 nm (OD_{440}) starts with values of 1.35, ranging from a minimum of 0.96 to a maximum of 1.57 (Table 16). This initial OD decreases during the testing period for 0.43 up to 1.19 OD-units to OD-values between 0.11 and 1.0. On average the initial OD decreases for 0.74 OD-units to values of 0.61 at t = 22hrs. The part of the decrease that is due to unspecific, non-phagocytosis dependent alterations contributes 0.13 OD-units of this total OD-decrease of 0.74 OD units. To obtain the pure phagocytic activity the OD-decrease of controls is corrected by the unspecific alterations, which results here in a mean phagocytosis activity (ΔOD_{corr}) of 0.62 OD-units within then testing period. This corresponds to an average percentage reduction of turbidity due to the engulfment of bacterial food of 47% which is almost the double of the predetermined minimum value of 25% (validity criterion 1). However, caused by the inherently variable, biological nature of sludge samples the phagocytic activity exhibits considerable differences with values as high as 79%. Note that the minimum phagocytic value in Table 16 is restricted – due to the validity criterion 1 it can't be less than 26%.

[11] On average the phagocytosis dependent OD-decrease ($\%\Delta OD_{corr}$) amounts to 47%. The minimum value of this decrease is set to 25%, otherwise the test run is considered invalid. According to the experience of the validation management this minimum is required to achieve reliable measurements.

Table 15: percent phagocytotic activity of all test runs (mean % ΔOD_{corr} of controls). Values below 25% are considerd to be invalid.

test substance	Laboratory No_run	mean % ΔOD_{corr} (controls)	criterion 1 fulfilled?
1-Octylamine	1_1	60%	yes
	2_1	44%	yes
	3_1	74%	yes
	4_1	40%	yes
	5_1	27%	yes
	5_2	14%	no
35-DCP	1_1	73%	yes
	2_1	44%	yes
	3_1	59%	yes
	4_1	40%	yes
	5_1	33%	yes
	5_2	24%	no
	5_3	23%	no
DMSO	1_1	79%	yes
	2_1	41%	yes
	3_1	61%	yes
	4_1	39%	yes
	5_1	26%	yes
	5_2	17%	no
hexachlorophene	1_1	45%	yes
	2_1	44%	yes
	3_1	57%	yes
	4_1	41%	yes
	5_1	18%	no
	5_2	27%	yes
phenyl ether	1_1	56%	yes
	2_1	42%	yes
	3_1	60%	yes
	4_1	50%	yes
	5_1	28%	yes
	5_2	29%	yes
valid runs only:	n valid	26	
	mean	47%	
	StDev	15%	
	median	44%	
	min	26%	
	max	79%	
	n (not valid)	5	

Table 16: Summary of the valid controls for all test runs.

	controls				
	2h_OD	22h_OD	ΔOD	ΔOD_{corr}	$\%\Delta OD_{corr}$
mean	1,35	0,61	0,74	0,62	47%
StDev	0,13	0,26	0,18	0,17	15%
median	1,36	0,66	0,74	0,61	44%
n	26	26	26	26	26
min	0,96	0,11	0,43	0,37	26%
max	1,57	1,01	1,19	1,03	79%

3.3 Defaunated controls

To verify non-phagocytosis related alterations of the optical density parallels including digitonin were run for each sample. According to the SOP, which served as the basis for the ring study, the maximum value for the unspecific decrease of these defaunated controls was limited to 25% (validity criterion 2, see also Figure 7). In all 31 test runs the OD-decrease of these fauna-free controls ($\%\Delta OD_{def}$) is below 15%. Thus, all runs fulfill that second criterion (Table 17).

For the defaunated controls the initial OD at t = 2 hours amounts on average to 1.45 (see Table 18). This is slightly higher (7%) than the average OD values of normal controls without digitonin (average initial OD = 1.35) . When testing for statistical difference, the P values provided by t-tests showed that the differences between both untreated and defaunated control OD-values are in most cases significant (23 runs out of 31). During the test period (2-22 hrs) the initial OD-values of defaunated controls decrease on average for 9% (0.13 OD-units) with a range between 4% and 14% to an OD of 1.33.

3.4 Controls for chemical effects on OD

Chemicals, especially at high concentrations, increase the turbidity of sludge samples. In defaunated samples, this unspecific OD-alteration can be recorded directly – uninfluenced by changes due to phagocytosis. According to the preliminary validity criteria, given in the ring test SOP, chemical concentrations leading to an increase of defaunated samples for more than 5% has to be excluded from data analyses (validity criterion 3).

In most cases (n = 22) the treatment group containing the highest chemical concentration complies with the validity criterion. However, in four test runs the highest concentration (C1) of the test chemicals octylamine, 3,5-DCP and phenyl ether was found to exceed this 5 percent increase[12] limit (Table 19) and thus curve fitting and EC_{50}-calculation were performed omitting these data. Based on the valid data (values greater than -5%) for the highest test concentration the average change of OD during the test period is close to zero. Nonetheless, this implies an average OD increase caused by the highest treatment group of around 9% relative to the chemical-free controls, taking into account that the average OD decrease of defaunated controls amounts to 9% (see Table 18).

[12] Note: whereas a decrease of OD (OD at t=2h exceeds OD at t=22hrs) is shown by positive values an increase is indicated by negative values (OD at t=2h is less than OD at t=22hrs).

Table 17: percent OD changes over the testing period (2 to 22 hrs) of defaunated controls (mean % ΔOD_{def} of controls) for all 31 test runs. Values below 25% are considered to be valid.

test substance	Laboratory No_run	mean % ΔOD_{def} (controls)	criterion 2 fulfilled?
1-Octylamine	1_1	8%	yes
	2_1	7%	yes
	3_1	9%	yes
	4_1	9%	yes
	5_1	4%	yes
	5_2	11%	yes
35-DCP	1_1	11%	yes
	2_1	7%	yes
	3_1	10%	yes
	4_1	9%	yes
	5_1	10%	yes
	5_2	9%	yes
	5_3	11%	yes
DMSO	1_1	11%	yes
	2_1	6%	yes
	3_1	11%	yes
	4_1	11%	yes
	5_1	7%	yes
	5_2	8%	yes
hexachlorophene	1_1	13%	yes
	2_1	7%	yes
	3_1	6%	yes
	4_1	11%	yes
	5_1	7%	yes
	5_2	6%	yes
phenyl ether	1_1	7%	yes
	2_1	7%	yes
	3_1	14%	yes
	4_1	9%	yes
	5_1	8%	yes
	5_2	7%	yes
valid runs only:	n valid	31	
	mean	9%	
	StDev	2%	
	median	8%	
	min	4%	
	max	14%	
	n (not valid)	0	

Table 18: statistics on average OD-values of defaunated controls.

	defaunated-controls			
	2h_OD_{def}	22h_OD_{def}	mean ΔOD_{def}	%ΔOD_{def}
mean	1,45	1,33	0,13	9%
StDev	0,07	0,08	0,03	2%
median	1,46	1,34	0,12	8%
n	26	26	26	26
min	1,34	1,20	0,06	4%
max	1,61	1,47	0,20	14%

Table 19: influence of the highest chemical concentration (C1) on OD-values. The given percent values reflect the difference in optical density between the initial 2 hour and the final 22 hour OD-value for each test run and the respective highest treatment group of defaunated parallels (mean % ΔOD_{def} (treatment C1). Values below -5% are regarded as invalid (validity criterion 3) and not included in the concentration-response calculation.

test substance	Laboratory No_run	mean % Δ OD_{def}(treatment C1)	criterion 3 fulfilled?
1-Octylamine	1_1	1%	yes
	2_1	-4%	yes
	3_1	-7%	no
	4_1	-4%	yes
	5_1	-10%	no
	5_2	-1%	yes
35-DCP	1_1	4%	yes
	2_1	-1%	yes
	3_1	-1%	yes
	4_1	-4%	yes
	5_1	-10%	no
	5_2	-3%	yes
	5_3	-1%	yes
DMSO	1_1	7%	yes
	2_1	4%	yes
	3_1	2%	yes
	4_1	6%	yes
	5_1	-1%	yes
	5_2	-3%	yes
hexachlorophene	1_1	4%	yes
	2_1	-3%	yes
	3_1	2%	yes
	4_1	0%	yes
	5_1	-4%	yes
	5_2	1%	yes
phenyl ether	1_1	1%	yes
	2_1	-2%	yes
	3_1	-11%	no
	4_1	-1%	yes
	5_1	6%	yes
	5_2	-2%	yes
valid runs only:	n valid	22	
	mean	0,1%	
	StDev	3%	
	median	-1%	
	min	-4%	
	max	7%	
	n (not valid)	4	

3.5 Concentration-response relationships

In total 31 tests were conducted. All five participating labs tested the five test substances. All laboratories succeeded in obtaining valid concentration-response curves for all 5 test chemicals. Five test runs of one laboratory had to be excluded as invalid due to an unusual low level of phagocytotic activity (see above, paragraph 'Controls').

Altogether, the results of 26 valid test runs were available, five for each of the four test chemicals octylamine, 3,5-DCP, DMSO and hexachlorophene and six for the test substance phenyl ether.

In one valid test run with the (volatile) substance phenyl ether, no effects could be observed over the whole concentration range. Consequently, no EC_{50}-value could be calculated. As a result, 25 EC_{50}-data served as the basis for the statistical evaluation of the ring test.

3.6 Effects of DMSO

Dimethyl sulfoxide is the preferred solvent in protozoan tests (Pauli & Berger, 1996). It provides good dissolution properties for a variety of organic chemicals and shows a low toxicity towards protozoa. In the ring test SOP dimethyl sulfoxide is considered to be the solvent of choice for poorly soluble organic substances. DMSO was also used to dissolve the highly lipophilic substance hexachlorophene. To verify possible toxic effects due to the use of this solvent, DMSO was included as model substance in the chemical test set.

Concentration response relationships could be recorded by all participants of the intercalibration exercise (Figure 8). The line in the diagram gives the best fit for all recorded mean effects (± StDev) of all participants. The dashed lines indicate the maximum allowable solvent concentration of 0.2% and the approximate NOEC- and LOEC-value. Ambiguous results were obtained in the displayed range between the NOEC and the LOEC. Here some participants find a significant response and some not. Below and above this concentration of about 0.5% and 1.2% DMSO a clearly dominant negative or positive effect was found (Table 20). According to the best fit function a DMSO concentration of 0.2% results in an inhibitory effect of 0.6% (i.e. < EC_{01}) and the actual level of effect at the NOEC and the LOEC is 3% (EC_{03}) and 14% (EC_{14}), respectively.

Table 20: NOEC and LOEC determination: mean effect values for all treatment groups of all test runs with DMSO and their statistically significant difference to the control (significance was calculated for each run on the basis of a Dunnett multiple comparison test); ns: not significant (p>5%);*: significant (P<5%); **: highly significant (P<1%,); green background: values below NOEC; white background: ambiguous results; red background: clearly dominant positive effects.

DMSO (v/v)	effect
0,1%	ns P>0.05
0,1%	ns P>0.05
0,2%	ns P>0.05
0,2%	ns P>0.05
0,2%	ns P>0.05
0,4%	ns P>0.05
0,5%	ns P>0.05
0,5%	ns P>0.05
0,5%	ns P>0.05
0,5%	* P<0.05
0,9%	** P<0.01
1,1%	ns P>0.05

DMSO (v/v)	effect
1,2%	** P<0.01
1,2%	** P<0.01
1,3%	** P<0.01
2,2%	** P<0.01
2,2%	ns P>0.05
3,1%	** P<0.01
3,1%	** P<0.01
3,3%	** P<0.01
4,4%	** P<0.01
5,5%	** P<0.01
7,6%	** P<0.01
7,7%	** P<0.01
8,3%	** P<0.01
8,7%	** P<0.01

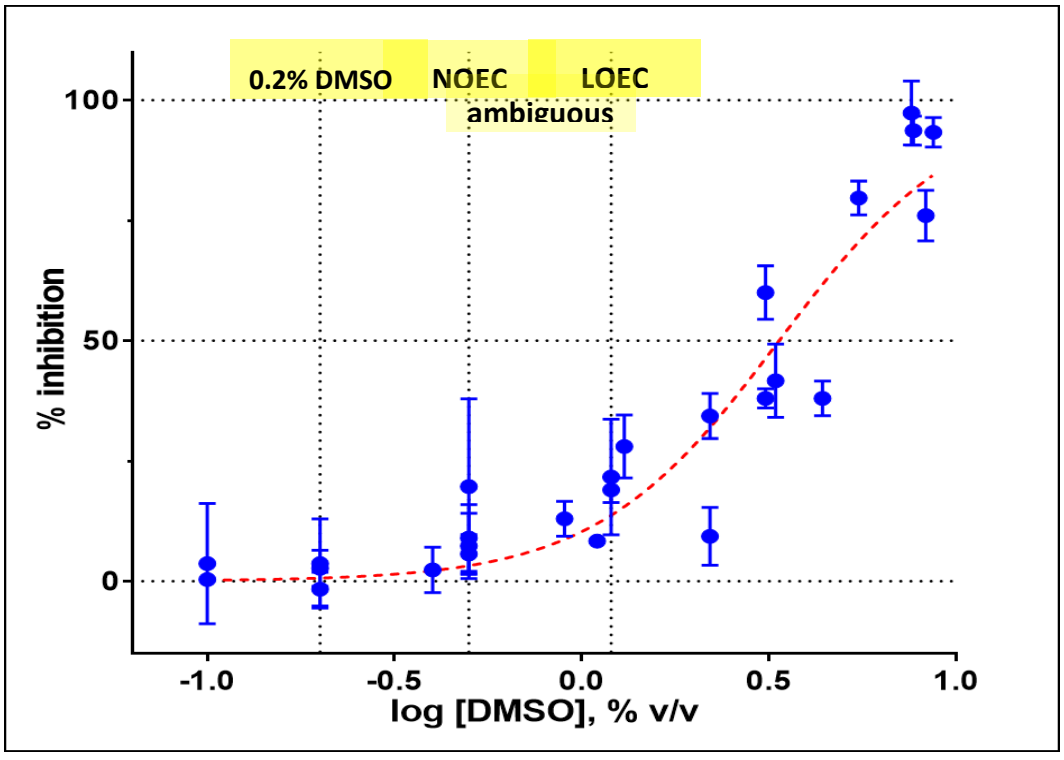

Figure 8: Effects of DMSO (v/v) on the phagocytic activity of activated sludge for all test runs. The symbols in the diagram represent the mean values of the respective treatment group for each test run (± StDev). The dotted line shows the best fit. The NOEC and the LOEC were calculated for each run on the basis of a Dunnett multiple comparison test. For further explanation, see text.

3.7 EC_{50}-values

For the statistical comparison concentration-response data from 25 test runs were available (five from each laboratory for each of the five test chemicals). Out of all recorded 31 test runs, five had to be rejected as invalid (phagocytotic activity below the validity criterion) and in one case no effect could be measured. In four test runs the highest test concentrations had to be excluded from data analysis due to failing the requirements of validity criterion 3 (chemical induced OD-increase).

The EC_{50}-values were calculated by non-linear curve fitting to log-transformed concentrations and normalized %inhibition values. In all but one test run data were successfully fitted to a symmetrical sigmoidal curve. In case of one test run good fit results could only be obtained by applying an asymmetric function.

Within the test set the EC_{50}-values cover more than four orders of magnitude varying from 1.3 mg/L in the case of hexachlorophene) and 3.1 g/L for dimethyl sulfoxide (mean values of all participants). In the following all EC_{50}-values (±95% confidence interval) are given for each test chemical.

3.7.1 Octylamine

The reported EC_{50} values for octylamine range from 14.9 to 46.4 mg/L with a ratio of highest to lowest value of 3.1 (Figure 9). The average EC_{50} across all laboratories for octylamine is 28.5 mg/L with a standard deviation of 6.1 mg/L and a variation coefficient (CV) of 48%. According to the Grubb's test there are no significant 'outliers' (P<0.05) in this data set:

1-Octylamine (mg/L)	
EC50-values (**inter-lab**)	
mean	28.5
St Error	6.1
median	24.6
St.Dev.	13.7
CV (%)	48%
variance	188.9
curtosis	-2.2
skewness	0.5
value range	31.6
minimum	14.9
maximum	46.4
ratio sample range	3.1
sum	142.6
number	5
± confidence (95.0%)	17.1

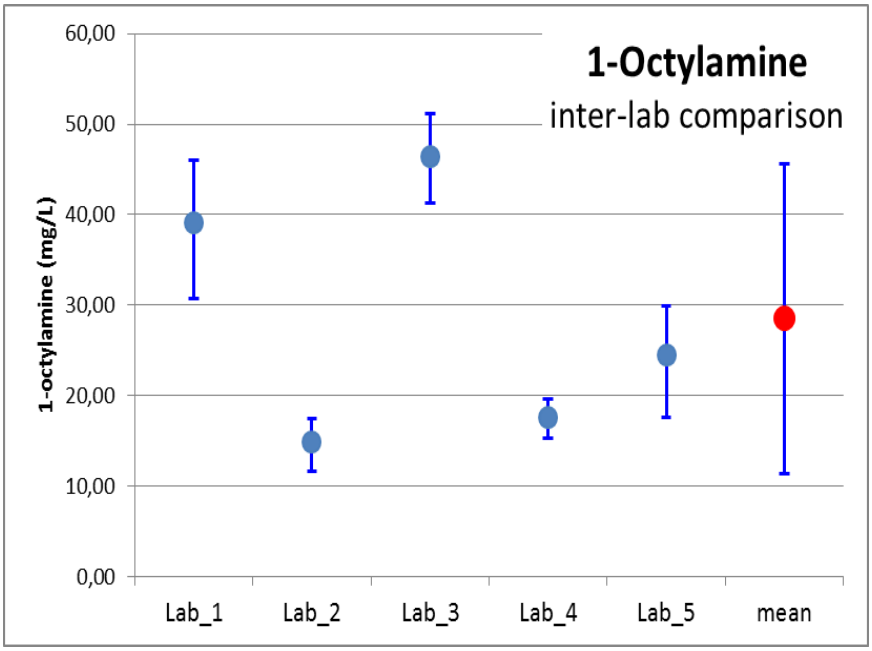

Figure 9: statistical data of the reported EC_{50}-values for 1-octylamine and their graphical comparison (±95% confidence limits).

3.7.2 3,5-Dichlorophenol

EC_{50}-values for 3.5-dichlorophenol (3,5-DCP) are between 1.49 and 5.13 mg/L with a ratio of highest to lowest of 3.45 (Figure 10). The mean EC_{50}-value of all five valid test runs is 2.91 mg/L with a standard deviation of 1.43 mg/L and a CV of 49%. No outliers could be detected by the Grubb's test:

3.5 DCP (mg/L)	
EC50-values(**inter-lab**)	
mean	2.91
St Error	0.64
median	2.32
St.Dev.	1.43
CV (%)	49%
variance	2.03
curtosis	0.75
skewness	1.10
value range	3.64
minimum	1.49
maximum	5.13
ratio sample range	3.45
sum	14.3
number	5
± confidence (95.0%)	1.77

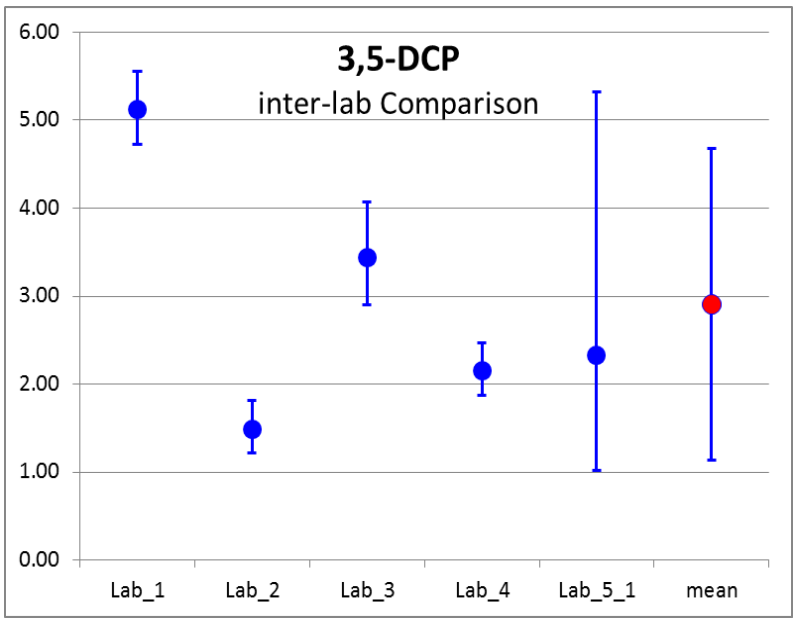

Figure 10: statistical data of the reported EC50-values for 3.5-dichlorophenol and their graphical illustration (±95% confidence limits).

3.7.3 Dimethyl sulfoxide (DMSO)

In case of DMSO the inter-lab comparison of EC_{50}-values shows a maximum deviation by a factor of 2.0 (22078 mg/L to 44219 mg/L). The mean EC_{50} amounts to 30810 mg/L with a standard deviation of 8353 mg/L and a CV of 27% (Figure 11). The Grubb's test shows no significant 'outliers' (P<0.05) in the data set.

DMSO (mg/L)	
EC50-values (**inter-lab**)	
mean	30810
St Error	3735
median	29747
St.Dev.	8353
CV (%)	27%
variance	69767096
curtosis	1.91
skewness	1.18
value range	22141
minimum	22078
maximum	44219
ratio sample range	2.0
sum	154051
number	5
± confidence (95.0%)	10371

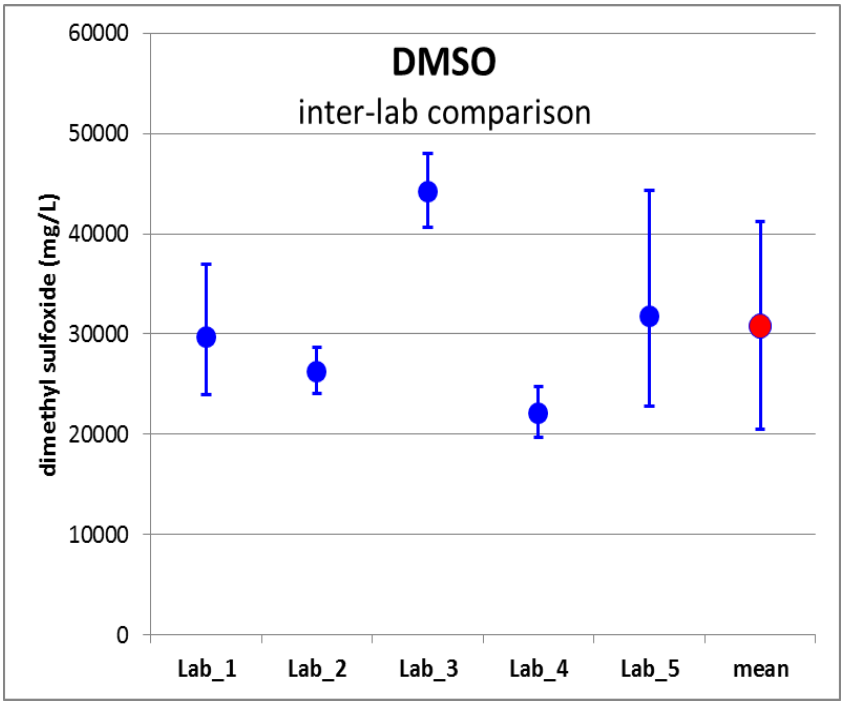

Figure 11: statistical comparison of the reported EC_{50}-values for dimethyl sulfoxide and documented as graphics (±95% confidence limits).

3.7.4 Hexachlorophene

The EC_{50}s range from 0.90 to 1.97 mg/L giving a ratio of highest to lowest of 2.2. The average EC_{50}-value of all laboratories is 1.34 mg/L with a standard deviation of 0.39 mg/L and a variation coefficient of 29% (Figure 12). In the data set no significant 'outlier' ($P<0.05$) can be detected by Grubb's test.

hexachlorophene (mg/L)	
EC50-values(**inter-lab**)	
mean	1.34
St Error	0.18
median	1.30
St.Dev.	0.39
CV (%)	29%
variance	0.15
curtosis	2.42
skewness	1.10
value range	1.08
minimum	0.90
maximum	1.97
ratio sample range	2.20
sum	6.72
number	5
± confidence (95.0%)	0.49

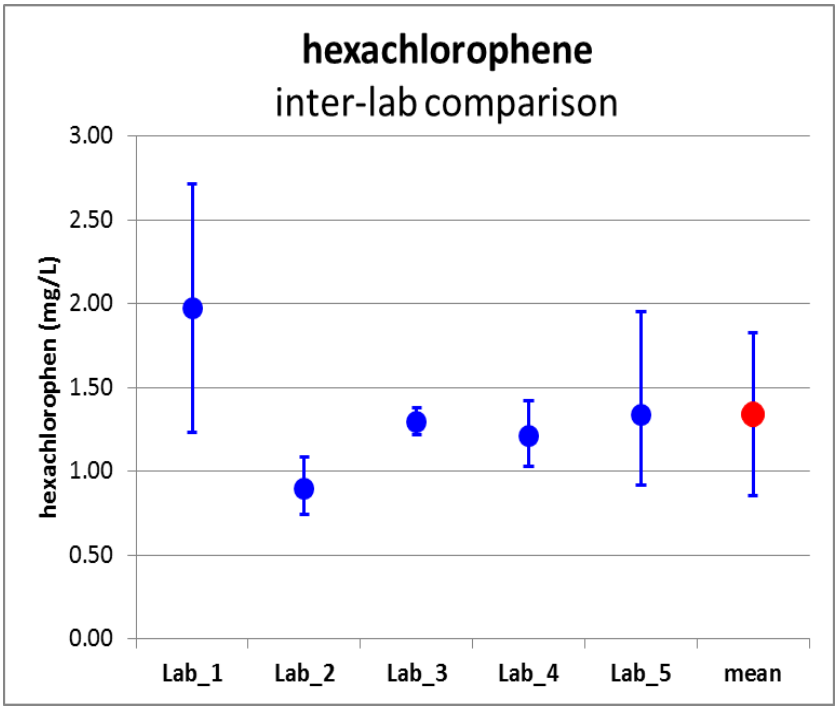

Figure 12: statistical information on EC_{50}-values from the ring study for the model substance hexachlorophene and its graphical presentation (±95% confidence interval).

3.7.5 Phenyl Ether

The reported EC_{50}-values for phenyl ether range from 7.39 mg/L to 16.7 mg/L with a ratio of highest to lowest of 2.26. The mean here is 12.5 mg/L and the standard deviation amounts to 3.68 mg/L giving a coefficient of variation of 29% (Figure 13). No outlier has been identified in the dataset by the Grubb's test.

phenyl ether (mg/L)	
EC50-values (**inter-Lab**)	
mean	12.5
St Error	1.65
median	14.0
St.Dev.	3.68
CV (%)	29%
variance	13.6
curtosis	-0.99
skewness	-0.53
value range	9.29
minimum	7.39
maximum	16.7
ratio sample range	2.26
sum	62.5
number	5
± confidence (95.0%)	4.57

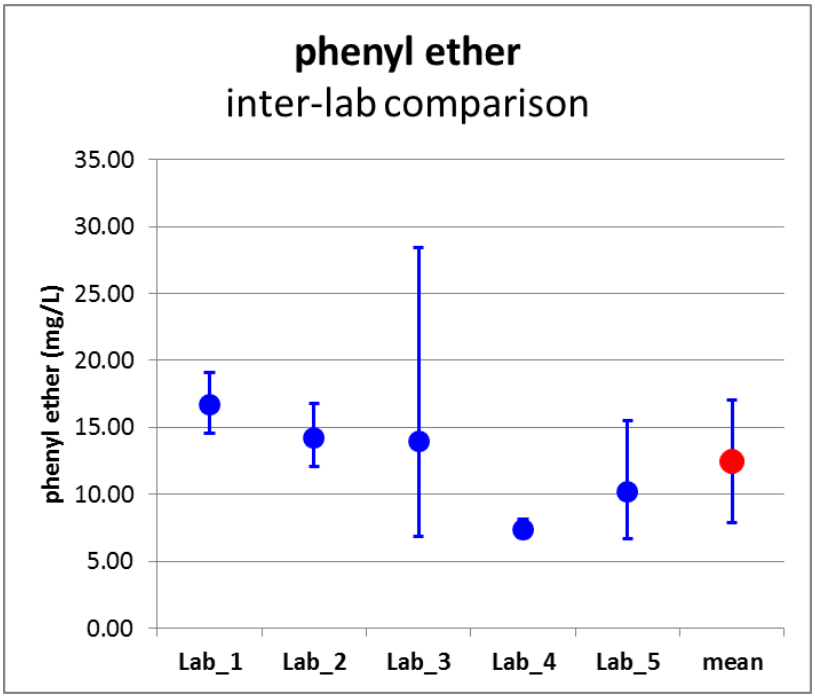

Figure 13: statistics on EC_{50}-values from the ring study for phenyl ether and its graphical presentation (±95% confidence interval).

3.7.6 Summary of the EC_{50} comparisons

Out of 31 recorded test runs 25 passed the validity criteria.

EC_{50}-data vary across laboratories on average by a factor of 2.60 with a mean coefficient of variation (CV) of 36%. The extreme values of the inter-lab EC_{50}-deviation range from 2-fold (DMSO) to 3.45-fold (3.5-DCP) with coefficients of variation in the range of 27% to 49% (see Table 21).

Table 21 summarizes the inter-laboratory deviations of the EC_{50}-values for all test chemicals.

	between (inter-) lab comparison			
	ratio highest to lowest	Coefficient of variance	Grubb's test	n
1-Octylamine	3.10	48%	no outlier	5
35-DCP	3.45	49%	no outlier	5
DMSO	2.00	27%	no outlier	5
hexachlorophene	2.20	29%	no outlier	5
phenyl ether	2.26	29%	no outlier	5
mean	2.60	36%		

4 DISCUSSION

4.1 Data basis

All EC_{50}-values were measured in sludge probes from different plants and in different labs. No valid data exist for intra-laboratory variability. Therefore no differentiated conclusions can be drawn with respect to intra- and exclusive inter-laboratory variability. However, the real situation of testing the phagocytic activity of native activated sludge implies not only tests in different labs but also continuously varying 'test organisms'. It might be reasonably assumed that in addition to inter-laboratory variability the differing sludge properties also play a dominant role in determining the variances of EC-values. Within-plant variations can occur from day to day as a result of shifts in the bacterial population. Sludge from different sources, and grown under different conditions, may also vary in response to inhibitors, because of varying degrees of reaction of some inhibitors with non-living sludge components. Therefore, in practice, EC_{50} values are also likely to be related to the specific activated sludge used for the phagocytosis test and inter- as well as intra-laboratory variations are only partly responsible for the scattering of effective concentrations.

Apart from pesticides, the majority of environmental chemicals are organic industrial chemicals, which were and are developed without considering their biological effects. About two thirds of them exert a type I (nonpolar) or type II (polar narcosis) mechanism of action (Veith, 2010). With the selection of three polar and two nonpolar narcotic chemicals for testing this main group of unspecific chemical mode of action is represented, although it has to be recognized that reactive and specifically acting chemicals[13] are missing in this investigation.

A biological test, however, should not only be able to detect these various effect mechanisms, the test protocol must also take into account the divergent physico-chemical properties of the compounds to be tested, making it possible to assess substances ranging from poorly soluble to highly volatile compounds. In choosing chemicals which cover a wide range of $logP_{ow}$-values and including volatile substances in the test set, some of the main problems associated with physicochemical properties of environmental chemicals were taken into consideration.

4.2 Controls

The concentration of the bacterial food suspension is chosen so that the food supply should not run out during the test period. After the addition of bacteria the fauna of the activated sludge phagocytose bacterial particles. This consumption is constant over time and can be followed by a linear decrease of OD[14]. The average value of the start OD after the addition of bacterial food is 1.35. This value lies in the linear range of the absorbance vs. *E.coli*-concentration. However for OD-values above 1.4 this correlation may no longer hold and the %effect calculation might slightly be affected. This may apply to about one third of all test runs (9 out of 26) and deserves further attention.

Phagocytosis stops at an OD of about 0.05 (data not shown) when the food runs out. The lowest recorded OD-value of all test runs after the 22 hours of incubation is 0.11 (minimum of 22hr-OD) indicating that in all test runs food supply is sufficient and phagocytosis can take place over the entire incubation period.

In order to be valid, it is necessary that mean phagocytotic activity (% ΔOD_{corr}(controls)) exceeds 25% within the testing time between 2 hours and 22 hours. The validation management assumes that an even higher value of 30% would be appropriate to further enhance the robustness without limiting the applicability of the test. Values for the phagocytotic activity (%ΔOD_{corr}) of activated sludge samples below 30% could only be observed in the laboratory which had no previous experience with activated sludge. Values below 30% are therefore considered exceptional and in most cases, appropriate experience with activated sludge provided, avoidable.

[13] For more information on classification, see, for example, overview of (Verhaar, et al., 1992; EPA White Paper, 2010).

[14] It should be noted that in the applied concentration range the amount of suspended bacteria is linearly related to the optical density at 440 nm up to around 1.4 OD-units

4.3 Defaunated controls

The start optical density of defaunated controls is in most cases (23 out of 31) significantly (on average 9%) higher than that of the faunated, normal controls. To defaunate the detergent digitonin is added at the start and remains in the sample throughout the entire test period. This ensures on the one hand a total inhibition of phagocytic activity, and on the other hand eliminates further working steps, thus facilitating test handling. However, the addition of digitonin is related to an increase of the optical density. Whereas eucaryotic sludge inhabitants are very effectively lysed in the presence of digitonin, there are no indications that the effects of digitonin are detrimental to the sludge bacteria. However digitonin may exert an effect on the sludge consistency due to its mild detergent action, resulting in a release of particles loosely bound to the sludge flocs. Both, the lysis of the fauna as well as the separation of particles from the flocs may result in the observed increase of turbidity and OD, respectively.

4.4 Controls for chemical effects on OD

The test chemicals have a tendency to increase the OD of the defaunated parallels. The defaunated parallels of each concentration step record these changes and help to correct the OD change due to phagocytosis.The chemical induced alterations can be seen if one compares the optical density of chemical containing defaunated parallels with the optical density of defaunated controls at the end of the test (Figure 14). With increasing concentration the optical density increases in relation to the control. This becomes also obvious when considering the percentage values of the optical density change of defaunated parallels at the end of the test. Whereas the defaunated controls show an average loss of OD of 9% during the test period, this amount of decrease diminishes in chemically treated defaunated parallels in a concentration-related manner. In some cases even an increase can be observed. Validity criterion 3 takes this chemical-induced change in OD into account and excludes concentrations leading to an increase of the optical density of more than 5% from data analyses. The 5% limit is an empirical value based on own measurements, taking into account that at even higher deviations of the optical density the effect calculation becomes inprecise and more and more uninterpretable.

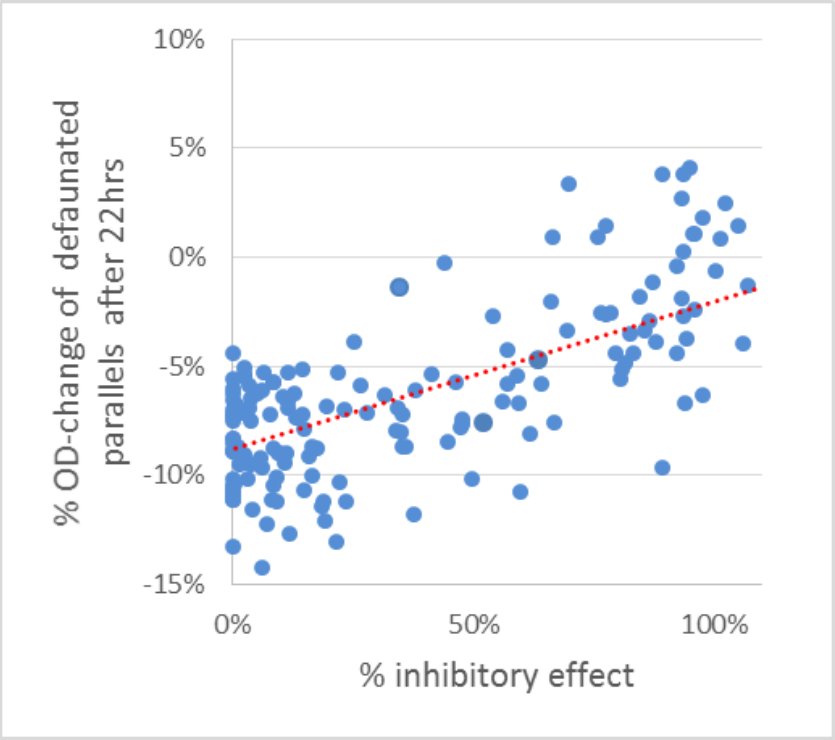

Figure 14: effect of chemicals on the final optical density. The figure illustrates the increase of the 22h-OD with increasing effect, i.e. chemical concentration, on the basis of data of defaunated controls. The 22h-OD of defaunated controls shows an average OD-loss of 9% (at 0% inhibition). With an increasing effect, i.e. with increasing chemical concentrations, the loss gets smaller and in some cases even an increase of the 22h-OD can be observed.The line shows the linear regression.

4.5 DMSO as potential solvent

According to the preliminary SOP dimethyl sulfoxide is the preferred organic solvent in case of poorly soluble compounds. In earlier studies it was found that up to concentrations of 0.2% (v/v) DMSO no significant effects on phagocytic activity could be observed. The same was the case in the present investigation. However, according to the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (OECD, 2000) the solvent concentration should be below 1/10th of the no observed effect concentration (NOEC) or in any case below 100 mg/l (or 0.1 mL/L). In the ring test a NOEC of 0.5% (v/v) was found. A tenth amounts to 0.5 mL/L DMSO, which is still substantially higher than 100 mg/L. According to the OECD Guideline the maximum allowable solvent concentration should therefore be reduced considerably in the final test protocol for the protozoan activated sludge test.

4.6 EC_{50}-values

One of the major criteria which a standardized test method must fulfil is reproducibility, independent of the laboratory or technician and – as in this particular case - independent of possible seasonal and local variations in

the 'test organism' activated sludge. In order to validate test systems it is therefore essential to compare toxic data from identical chemicals tested in several laboratories with the same test protocol. Although the data presented in this report only reflect comparisons for a small number of test chemicals and a small number of participants, this interlaboratory study provides some important information about the degree of standardization of the protozoan activated sludge test protocol.

In the present study for all of the five test chemicals concentration-response curves could be obtained over the whole effect range. With the exception of one case R-squared-values above 0.85 indicate that the model of a S-shaped curve fits well the collected data. The 95%-confidence intervals of the best fit deviate from the EC_{50}-value in 23 out of the 25 cases at maximum by a factor of less than 2.5. Only in two test runs a higher deviation of up to 5-fold could be observed.

4.7 Interlaboratory comparisons

No significant outliers in the data set provided by the different labs using sludge from different wastewater treatment plants were found by the Grubbs test. Based on the preliminary test protocol the EC_{50}-data from each participant deviate by not more than a factor of two from the interlaboratory mean. The observed coefficients of variations between 30% and 50% correspond well to to between-laboratory %CVs found for fully standardized single species ecotoxicity tests (e.g. Microtox: 30-55% (Na ŁJaŒecki, et al., 2010), *Daphnia*: around 40% in 80% of the reviewed round robin data (Rue, et al., 1988)) and even to within-laboratory variations of the OECD respiration inhibition test (intra-laboratory %CVs of 30-40% for 3,5-DCP (Gendig, et al., 2003)).

4.8 EC_{50}s and lipophilicity

As can be seen from Figure 15, the measured effects are in good agreement with the known effect profile of the substances: The polar narcotic compounds octylamine and 3,5-dichlorophenol show a more than 10-times higher toxicity (dots lying below the line) than the rest of the substances with nonpolar narcotic effects like DMSO and phenyl ether or with a very high lipophilicity as in the case of hexachlorophene. The dots representing the logEC_{50}-data for the chemicals DMSO, phenyl ether and hexachlorophene were connected with a straight line, which should reflect the so-called basis or minimal toxicity.

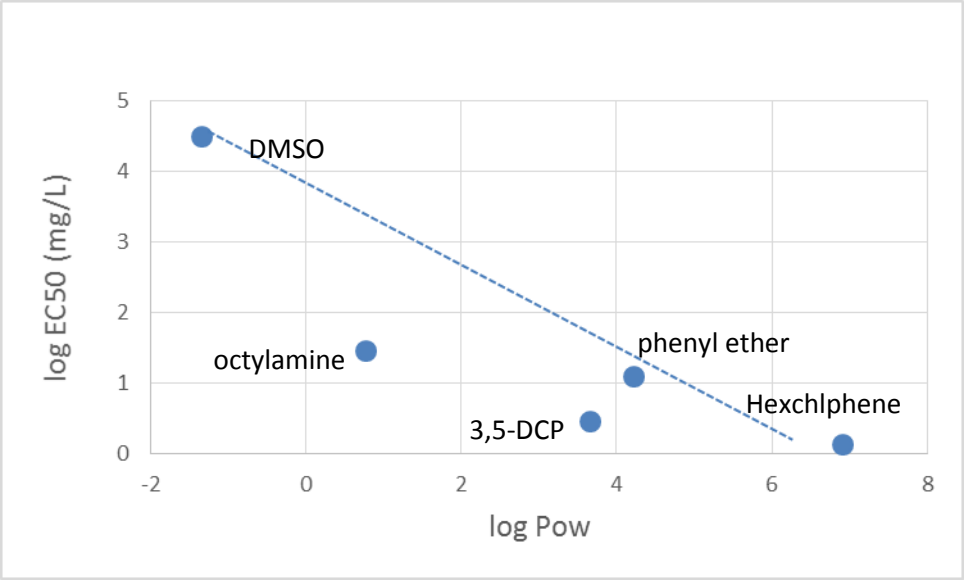

Figure 15: log EC_{50}-data of the test chemicals versus their log octanol-water partition coefficient.The dotted line illustrates the supposed baseline toxicity caused by nonpolar narcosis.

4.9 Comparison with toxicological data from other ciliate and activated sludge tests

For each of the five ring test chemicals, toxicological data for ciliate growth and phagocytosis were available. Data from the bacterial respiration inhibition test with activated sludge (OECD No. 209) were only found for three of the test chemicals (Table 22).

Comparison of the results of the EC_{50} value with the protozoan and the bacteria activated sludge test (OECD 209) demonstrates that the protozoa are more susceptible to n-octylamine and 3,5-dichlorophenol (with a factor of 6 and higher) and markedly less sensitive to dimethyl sulfoxide (by a factor of more than 2 orders).

The comparison of the EC_{50} values for the phagocytosis in activated sludge and the growth and phagocytosis of the ciliate Tetrahymena reveals no marked differences in their relative sensitivity to the test chemicals (Figure 16). EC_{50}s of the Tetrahymena growth and the protozoan activated sludge test deviate by a factor of less than five and the EC_{50}s of the phagocytosis assays with activated sludge and Tetrahymena by a factor of less than two. This is all the more striking because the ciliate tests are performed with a single species and in a medium with a comparably low organic-matter level of around one tenth of that of the protozoan activated sludge test. Furthermore the ciliate tests with Tetrahymena record in the case of the growth assay a multi-generation response to the chemicals and in the case of the phagocytosis assay a short time response within a few minutes. Significant differences in the sensitivity to a toxicant correlated with the amount of organic content in the medium may be expected for especially reactive chemicals and for heavy metals. Further studies will have to decide whether this similarity in sensitivity is also valid for a broader range of environmental chemicals.

Table 22: comparison of the ring test data with EC_{50}-values of the OECD 209 activated sludge respiration inhibition test and growth and phagocytosis tests with the ciliate Tetrahymena. References are given in the Table.

Chemical	Protozoan activated sludge (this study)	OECD 209: AS resp.inhib test	Tetrahymena growth	Tetrahymena phagocytosis
n-octylamine	28.5 (15-46) mg/L	>200 mg/L [21]	140 mg/L*	15 mg/L*
3,5-DCP	2.91 (1.5-5) mg/L	5-30 mg/L [22]	4.4 mg/L [10]	n.a.
DMSO	30.8 (22-44) g/L	10-100 mg/L [23]	10 g/L*	18 g/L*
hexachlorophene	1.34 (0.9-2) mg/L	n.a.	0.3 mg/L*	1.2 mg/L*
Phenyl ether	12.5 (7.4-17) mg/L	n.a.	34 mg/L*	16 mg/L*

*) own measurements (not published)

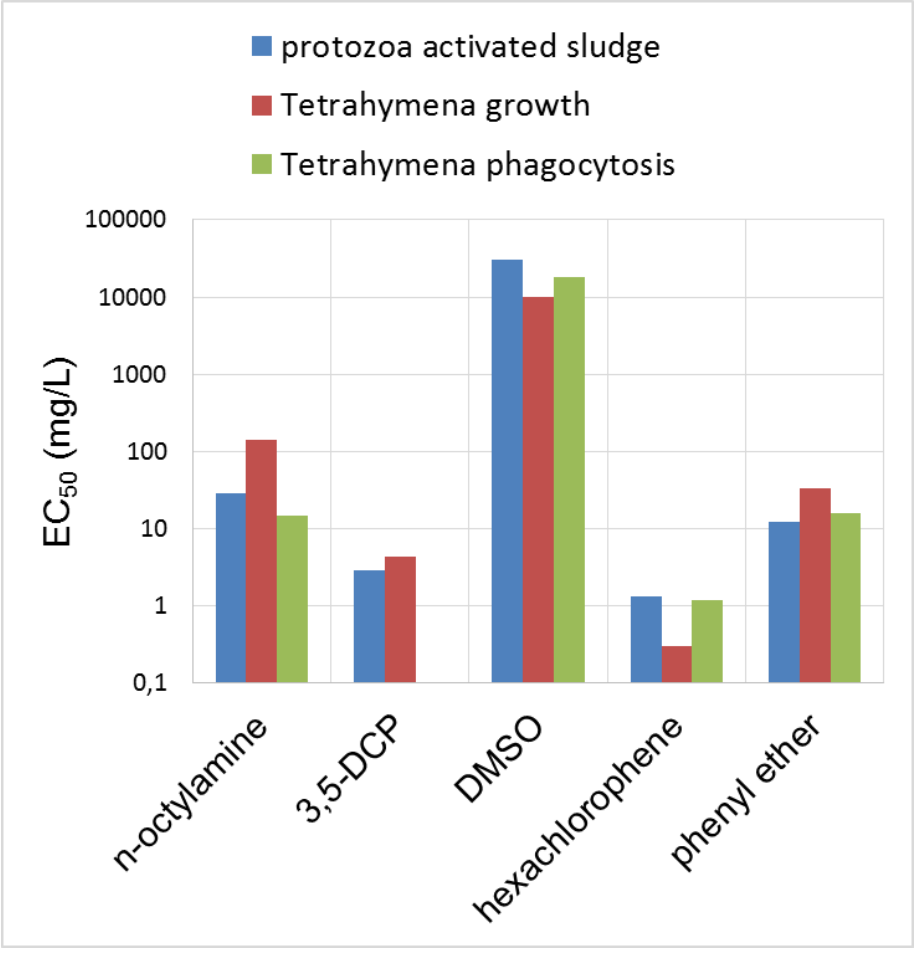

Figure 16: comparison of EC_{50}s from the protozoan activated sludge test and from *Tetrahymena* growth and phagocytosis assays.

5 CONCLUSION

A key prerequisite for successful testing is a high phagocytic activity of the activated sludge. This important biological characteristic can be usually inquired by the local wastewater authorities. The validation management therefore proposes that the validity criterion 1 should be raised from 25% to 30%.

The optical density at the start of the test is in some cases very close to the point at which no clear linear correlation exists any more between substrate concentration and optical density. The validation management, therefore, proposes to reduce in parallel both substrate as well as activated sludge concentration for 10% from 4 g/L and 1 g/L to values of 0.36 mg/L and 0.9 g/L, respectively.

Taking into account that four of the five participating laboratories had no previous experience with protozoa testing and that activated sludge tests are expected to vary considerably more than those from tests with well defined test organisms the outcome of the ring test indicates an already relatively high degree of standardization of the test protocol: All laboratories obtained valid concentration response relationships for all model chemicals without previous training. Non-valid test runs can be mainly attributed to low phagocytic activity of the particular sludge batch. The observed inter-laboratory coefficients of variation of the EC_{50}-values of around 40% are within the normal range of fully standardized single species ecotoxicity tests.

The protozoan activated sludge test seems to have a different toxic profile as compared to the bacterial respiration inhibition test with activated sludge (OECD No. 209). It infers that these different functional groups supplement, rather than replace, each other with regard to the estimation of toxic effects in waste water treatment. However, a high degree of correspondence is found with growth and phagocytosis tests of the ciliate *Tetrahymena*.

6 ACKNOWLEDGEMENTS

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- Chemicals Evaluation and Research Institute / CERI. Section 1

  3-2-7 Miyanojin. Kurume. Fukuoka 839-0801. Japan

- DR.U.NOACK-LABORATORIEN

  Käthe-Paulus-Straße 1. D-31157 Sarstedt. Germany

- Eurofins Agroscience Services EcoChem GmbH. Aquatic Toxicology

  Eutinger Str. 24. 75223 Niefern-Öschelbronn. Germany

- Umweltbundesamt. Wassergefährdende Stoffe. Ökotoxikologielabor

  Schichauweg 58. 12307 Berlin. Germany

7 APPENDIX I – Calculated & original data

7.1 Controls

untreated and defaunated

(digitonin-treated) controls

		controls:						Digitonin-controls:					
Lab	run	2h_OD	22h_OD	$\Delta OD_{Control}$	mean ΔOD_{corr}		%OD-decrease	2h_OD	22h_OD	mean ΔOD		%OD-decrease	
Lab_1	1_1	1.27	0.39	0.88	0.75	✓	60%	1.46	1.34	0.122	✓	8%	
Lab_1	1_1	1.26	0.18	1.08	0.92	✓	73%	1.42	1.26	0.157	✓	11%	
Lab_1	1_1	1.30	0.11	1.19	1.03	✓	79%	1.51	1.34	0.167	✓	11%	
Lab_1	1_1	1.38	0.57	0.82	0.62	✓	45%	1.50	1.30	0.198	✓	13%	
Lab_1	1_1	1.30	0.46	0.84	0.73	✓	56%	1.53	1.42	0.112	✓	7%	
Lab_2	2_1	1.34	0.69	0.65	0.58	✓	44%	1.50	1.39	0.107	✓	7%	
Lab_2	2_1	1.34	0.65	0.69	0.59	✓	44%	1.46	1.36	0.102	✓	7%	
Lab_2	2_1	1.40	0.73	0.67	0.57	✓	41%	1.52	1.42	0.095	✓	6%	
Lab_2	2_1	1.36	0.66	0.70	0.60	✓	44%	1.50	1.40	0.098	✓	7%	
Lab_2	2_1	1.33	0.68	0.65	0.55	✓	42%	1.47	1.37	0.098	✓	7%	
Lab_3	3_1	0.96	0.13	0.83	0.71	✓	74%	1.36	1.24	0.120	✓	9%	
Lab_3	3_1	1.27	0.38	0.89	0.75	✓	59%	1.34	1.21	0.137	✓	10%	
Lab_3	3_1	1.22	0.33	0.89	0.75	✓	61%	1.35	1.20	0.145	✓	11%	
Lab_3	3_1	1.46	0.55	0.91	0.82	✓	57%	1.44	1.35	0.087	✓	6%	
Lab_3	3_1	1.10	0.25	0.85	0.66	✓	60%	1.39	1.20	0.188	✓	14%	
Lab_4	4_1	1.57	0.81	0.76	0.62	✓	40%	1.61	1.47	0.137	✓	9%	
Lab_4	4_1	1.55	0.80	0.76	0.62	✓	40%	1.57	1.43	0.140	✓	9%	
Lab_4	4_1	1.40	0.70	0.69	0.54	✓	39%	1.40	1.24	0.155	✓	11%	
Lab_4	4_1	1.40	0.67	0.73	0.58	✓	41%	1.40	1.25	0.152	✓	11%	
Lab_4	4_1	1.47	0.61	0.86	0.73	✓	50%	1.48	1.34	0.135	✓	9%	
Lab_5	5_1	1.36	0.93	0.43	0.37	✓	27%	1.36	1.30	0.060	✓	4%	
Lab_5	5_2	1.34	0.99	0.35	0.18	✗	14%	1.48	1.32	0.165	✓	11%	not valid control
Lab_5	5_1	1.35	0.77	0.59	0.44	✓	33%	1.37	1.23	0.143	✓	10%	
Lab_5	5_2	1.39	0.93	0.47	0.34	✗	24%	1.43	1.30	0.127	✓	9%	not valid control
Lab_5	5_3	1.44	0.96	0.48	0.33	✗	23%	1.47	1.32	0.155	✓	11%	not valid control
Lab_5	5_1	1.52	1.01	0.51	0.40	✓	26%	1.52	1.41	0.113	✓	7%	
Lab_5	5_2	1.45	1.08	0.37	0.24	✗	17%	1.52	1.40	0.127	✓	8%	not valid control
Lab_5	5_1	1.44	1.08	0.36	0.26	✗	18%	1.45	1.34	0.107	✓	7%	not valid control
Lab_5	5_2	1.42	0.96	0.46	0.38	✓	27%	1.49	1.41	0.083	✓	6%	
Lab_5	5_1	1.45	0.93	0.52	0.41	✓	28%	1.44	1.33	0.112	✓	8%	
Lab_5	5_2	1.43	0.92	0.52	0.41	✓	29%	1.47	1.37	0.102	✓	7%	
	mean	1.36	0.61	0.74	0.62		47%	1.46	1.33	0.13		9%	
	StDev	0.12	0.26	0.18	0.17		15%	0.07	0.07	0.03		2%	
	CV %	9%	43%	25%	27%		32%	4%	6%	25%		26%	
	media	1.38	0.61	0.74	0.62		47%	1.47	1.34	0.13		9%	
	n	31	26	26	26		26	31	31	31		31	
	min	0.96	0.11	0.43	0.37		26%	1.34	1.20	0.06		4%	
	min	1.57	1.01	1.19	1.03		79%	1.61	1.47	0.20		14%	

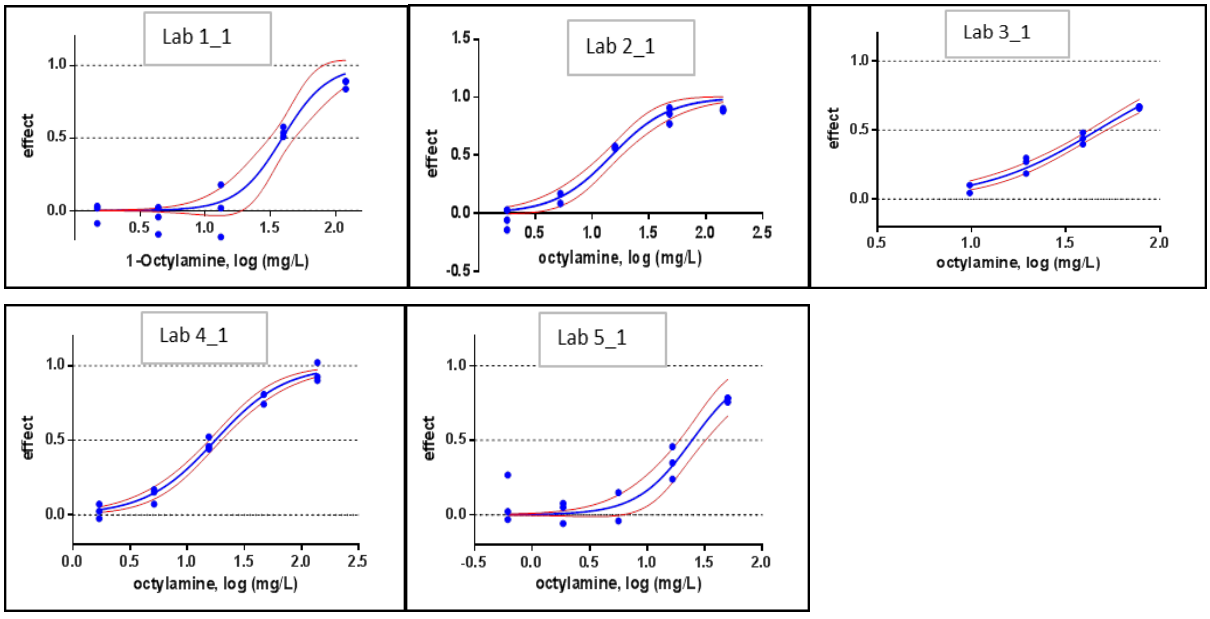
7.2 Chemical effects, fitting model & EC_{50}-values

Lab	run	test substance	highest tested conc.	effect at highest conc.	lowest tested conc.	effect at lowest conc.	single values fitting model	r2	EC50 (mg/L)	lower Conf.limit	upper Conf.limit	control %OD-decrease		Digitonin-control %OD-decrease		%OD-change C1+		remarks
Lab_1	1_1	1-Octylamine	119.08	87%	1.47	-6%	sigmoidal	0.938	39.2	32.24	47.58	✓	60%	✓	8%	✓	1%	
Lab_1	1_1	35-DCP	14.04	92%	0.17	0%	sigmoidal	0.987	5.13	4.73	5.56	✓	73%	✓	11%	✓	4%	
Lab_1	1_1	DMSO	70222	94%	1798	3%	sigmoidal	0.916	29747	23909	37011	✓	79%	✓	11%	✓	7%	
Lab_1	1_1	hexachlorophene	9.86	89%	0.12	-5%	sigmoidal	0.954	1.97	1.23	2.72	✓	45%	✓	13%	✓	4%	
Lab_1	1_1	phenyl ether	66.3	100%	0.82	2%	sigmoidal	0.977	16.7	14.56	19.11	✓	56%	✓	7%	✓	1%	
Lab_2	2_1	1-Octylamine	142.24	89%	1.76	-6%	sigmoidal	0.962	14.87	12.21	18.10	✓	44%	✓	7%	✓	-4%	
Lab_2	2_1	35-DCP	14.2	96%	0.18	12%	sigmoidal	0.961	1.49	1.22	1.81	✓	44%	✓	7%	✓	-1%	
Lab_2	2_1	DMSO	50153	80%	1284	1%	sigmoidal	0.984	26232	24040	28623	✓	41%	✓	6%	✓	4%	
Lab_2	2_1	hexachlorophen	10.67	93%	0.13	11%	sigmoidal	0.964	0.898	0.744	1.08	✓	44%	✓	7%	✓	-3%	
Lab_2	2_1	phenyl ether	50.15	102%	0.62	8%	sigmoidal	0.959	14.25	12.1	16.79	✓	42%	✓	7%	✓	-2%	
Lab_3	3_1	1-Octylamine	157.01	70%	9.81	6%	sigmoidal	0.969	46.43	41.75	51.64	✓	74%	✓	9%	✗	-7%	C1 not valid
Lab_3	3_1	35-DCP	15.7	96%	0.98	19%	sigmoidal	0.933	3.43	2.90	4.07	✓	59%	✓	10%	✓	-1%	
Lab_3	3_1	DMSO	79203	93%	4950	6%	sigmoidal	0.967	44219	40683	48061	✓	61%	✓	11%	✓	2%	
Lab_3	3_1	hexachlorophene	11.86	96%	0.74	23%	sigmoidal	0.987	1.30	1.22	1.38	✓	57%	✓	6%	✓	2%	
Lab_3	3_1	phenyl ether	79.2	120%	4.95	8%	sigmoidal	0.360	13.97	6.87	28.40	✓	60%	✓	14%	✗	-11%	C1 not valid
Lab_4	4_1	1-Octylamine	138.86	95%	1.71	2%	sigmoidal	0.986	17.58	15.54	19.90	✓	40%	✓	9%	✓	-4%	
Lab_4	4_1	35-DCP	13.89	93%	0.17	2%	sigmoidal	0.982	2.15	1.88	2.47	✓	40%	✓	9%	✓	-4%	
Lab_4	4_1	DMSO	69429	98%	1777	-2%	sigmoidal	0.979	22078	19708	24734	✓	39%	✓	11%	✓	6%	
Lab_4	4_1	hexachlorophene	10.41	94%	0.13	0%	sigmoidal	0.975	1.21	1.03	1.42	✓	41%	✓	11%	✓	0%	
Lab_4	4_1	phenyl ether	77.59	101%	0.96	1%	sigmoidal	0.991	7.39	6.73	8.11	✓	50%	✓	9%	✓	-1%	
Lab_5	5_1	1-Octylamine	150.55	83%	0.62	9%	sigmoidal	0.898	24.55	19.13	31.51	✓	27%	✓	4%	✗	-10%	C1 not valid
Lab_5	5_2	1-Octylamine	155.18	162%	0.64	-44%	sigmoidal	0.7441	~ 16,74	(Very wide)	(Very wide)	✗	14%	✓	11%	✓	-1%	not valid control
Lab_5	5_1	35-DCP	15.91	130%	0.07	-3%	asymsig	0.893	2.32	1.01	5.33	✓	33%	✓	10%	✗	-10%	C1 not valid
Lab_5	5_2	35-DCP	15.52	117%	0.06	6%	sigmoidal	0.929	2.35	1.80	3.07	✗	24%	✓	9%	✓	-3%	not valid control
Lab_5	5_3	35-DCP	15.05	105%	0.06	2%	sigmoidal	0.832	1.85	1.19	2.87	✗	23%	✓	11%	✓	-1%	not valid control
Lab_5	5_1	DMSO	75272.73	76%	770.79	4%	sigmoidal	0.860	31775	22750	44381	✓	26%	✓	7%	✓	-1%	
Lab_5	5_2	DMSO	75272.73	70%	770.79	16%	sigmoidal	0.754	11155	6748	18440	✗	17%	✓	8%	✓	-3%	not valid control
Lab_5	5_1	hexachlorophene	11.58	127%	0.05	18%	sigmoid	0.841	~ 1,169	(Very wide)	(Very wide)	✗	18%	✓	7%	✓	-4%	not valid control
Lab_5	5_2	hexachlorophene	10.53	107%	0.04	-17%	sigmoidal	0.887	1.34	0.92	1.96	✓	27%	✓	6%	✓	1%	
Lab_5	5_1	phenyl ether	77.59	9%	0.32	9%	sigmoidal	-	-	-	-	✓	28%	✓	8%	✓	6%	no effect!
Lab_5	5_2	phenyl ether	70.22	98%	0.29	-16%	sigmoidal	0.867	10.18	6.68	15.52	✓	29%	✓	7%	✓	-2%	
n (tested)			31				n (EC50s)		25									
n (valid)			26				n (valid)		26									

7.3 Curve-fitting statistics, best fits[15]

curve fit data: 1-octylamine

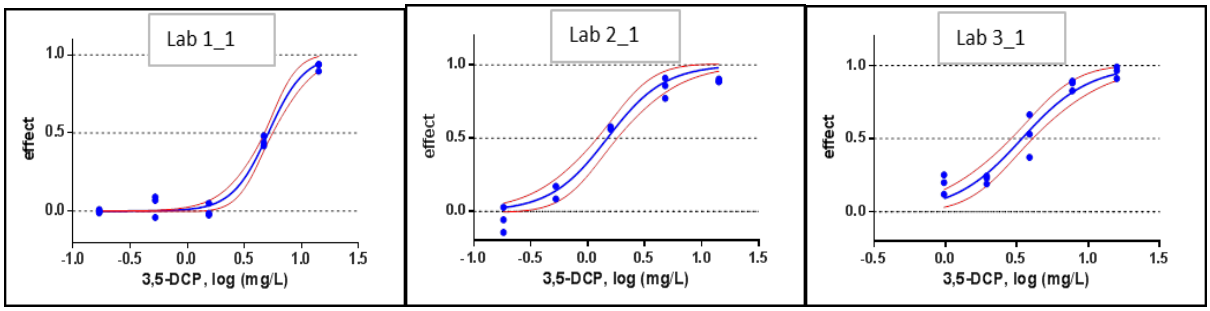
	Lab 1_1	Lab 2_1	Lab 3_1	Lab 4_1	Lab 5_1
type of fitting curve	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)
Best-fit values					
Top	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Bottom	= 0,0	= 0,0	= 0,0	= 0,0	= 0,0
LogIC50	1.593	1.172	1.667	1.245	1.39
HillSlope	2.505	1.728	1.417	1.427	1.815
IC50	39.16	14.87	46.43	17.58	24.55
Span	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Std. Error					
LogIC50	0.03913	0.03953	0.02072	0.02484	0.05018
HillSlope	0.676	0.2484	0.1087	0.1044	0.3421
95% Confidence Intervals					
LogIC50	1,508 to 1,677	1,087 to 1,258	1,621 to 1,713	1,191 to 1,299	1,282 to 1,498
HillSlope	1,044 to 3,965	1,192 to 2,265	1,175 to 1,659	1,202 to 1,653	1,076 to 2,555
IC50	32,24 to 47,58	12,21 to 18,10	41,75 to 51,64	15,54 to 19,90	19,13 to 31,51
Goodness of Fit					
Degrees of Freedom	13	13	10	13	13
R square	0.9376	0.9617	0.9694	0.9859	0.898
Absolute Sum of Squares	0.1362	0.08548	0.01872	0.02752	0.1379
Sy.x	0.1024	0.08109	0.04326	0.04601	0.103
Constraints					
Top	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000
Bottom	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0
Number of points					
Analyzed	15	15	12	15	15

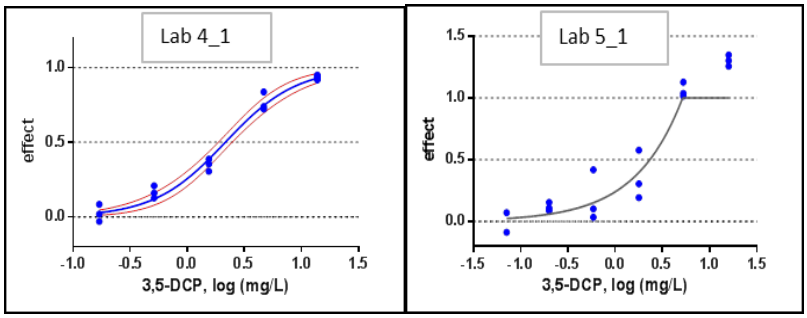


[15] Decription of the test runs: No of participant and No of test run (e.g. Lab 1_1: lab1, test run 1)

curve fit data: 3,5-DCP

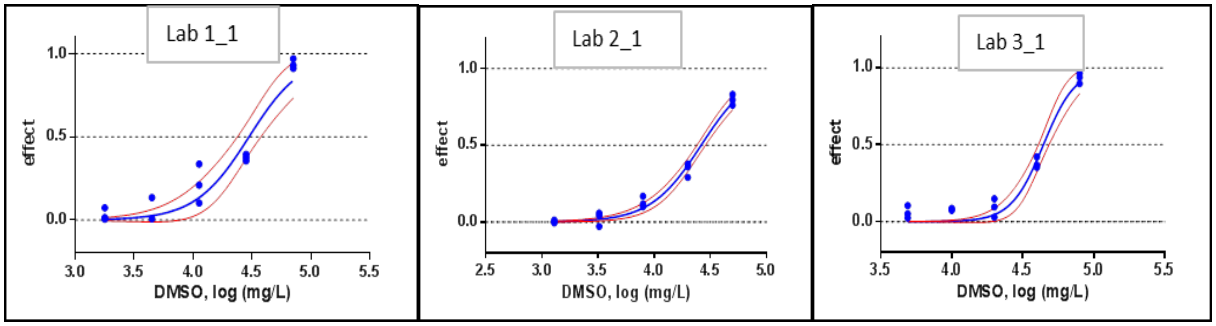
	Lab 1_1	Lab 2_1	Lab 3_1	Lab 4_1	Lab 5_1
type of fitting curve	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Asym. sig., 5PL, X is log(conc.)
Best-fit values					
Top	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Bottom	= 0,0	= 0,0	= 0,0	= 0,0	= 0,0
LogIC50	0.7098	0.1724	0.5358	0.3332	0.3663
HillSlope	2.747	1.733	1.839	1.391	~ 114,2
IC50	5.126	1.487	3.434	2.154	2.324
Span	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Std. Error					
LogIC50	0.01625	0.03957	0.03404	0.02772	0.169
HillSlope	0.3581	0.2498	0.245	0.1103	~ 5273/ S ~ 0,3538
95% Confidence Intervals					
LogIC50	0,6747 to 0,745	0,08688 to 0,2578	0,4623 to 0,6094	0,2733 to 0,3931	0,006110 to 0,7264
HillSlope	1,973 to 3,520	1,194 to 2,273	1,310 to 2,368	1,153 to 1,629	de) / S (Very wide)
IC50	4,728 to 5,558	1,221 to 1,811	2,899 to 4,068	1,876 to 2,472	1,014 to 5,326
Goodness of Fit					
Degrees of Freedom	13	13	13	13	15
R square	0.9871	0.9614	0.9329	0.982	0.8928
Absolute Sum of Squares	0.02549	0.08597	0.1053	0.03327	0.5055
Sy.x	0.04428	0.08132	0.09	0.05059	0.1836
Constraints					
Top	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000
Bottom	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0
Number of points					
Analyzed	15	15	15	15	18

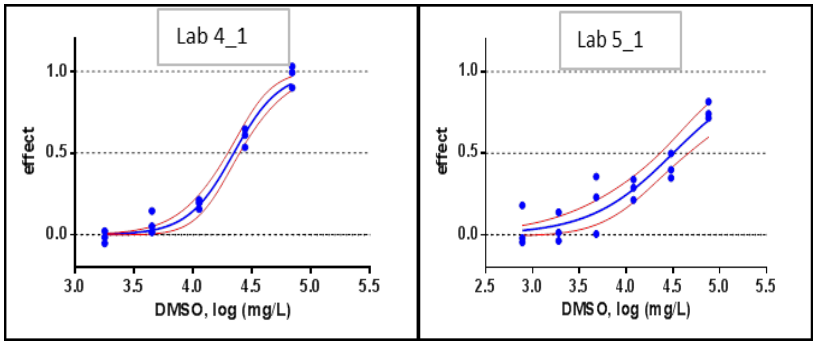




curve fit data: DMSO

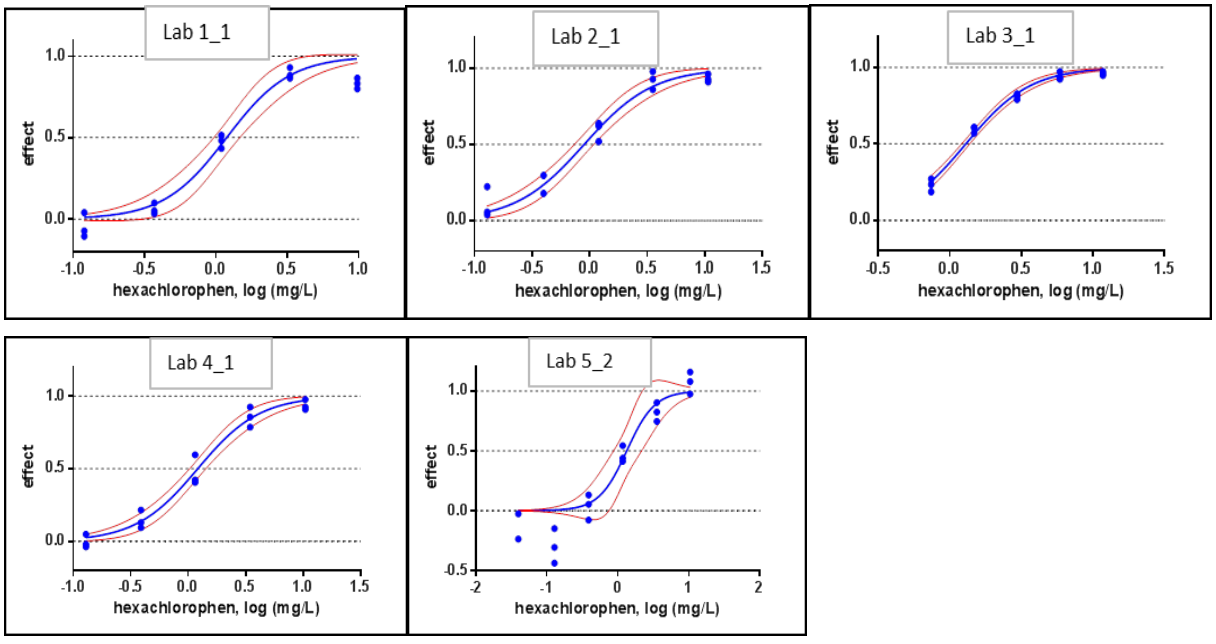
	Lab 1_1	Lab 2_1	Lab 3_1	Lab 4_1	Lab 5_1
type of fitting curve	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)
Best-fit values					
Top	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Bottom	= 0,0	= 0,0	= 0,0	= 0,0	= 0,0
LogIC50	4.473	4.419	4.646	4.344	4.502
HillSlope	1.91	1.932	3.823	2.229	0.977
IC50	29747	26232	44219	22078	31775
Span	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Std. Error					
LogIC50	0.04392	0.01754	0.01675	0.02283	0.06845
HillSlope	0.3477	0.1419	0.5724	0.2346	0.1585
95% Confidence Intervals					
LogIC50	4,379 to 4,568	4,381 to 4,457	4,609 to 4,682	4,295 to 4,393	4,357 to 4,647
HillSlope	1,159 to 2,661	1,625 to 2,238	2,586 to 5,059	1,722 to 2,736	0,6409 to 1,313
IC50	23909 to 37011	24040 to 28623	40683 to 48061	19708 to 24734	22750 to 44381
Goodness of Fit					
Degrees of Freedom	13	13	13	13	16
R square	0.9161	0.9836	0.9673	0.9789	0.8599
Absolute Sum of Squares	0.1372	0.02154	0.05507	0.04425	0.1745
Sy.x	0.1027	0.04071	0.06509	0.05834	0.1044
Constraints					
Top	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000
Bottom	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0
Number of points					
Analyzed	15	15	15	15	18





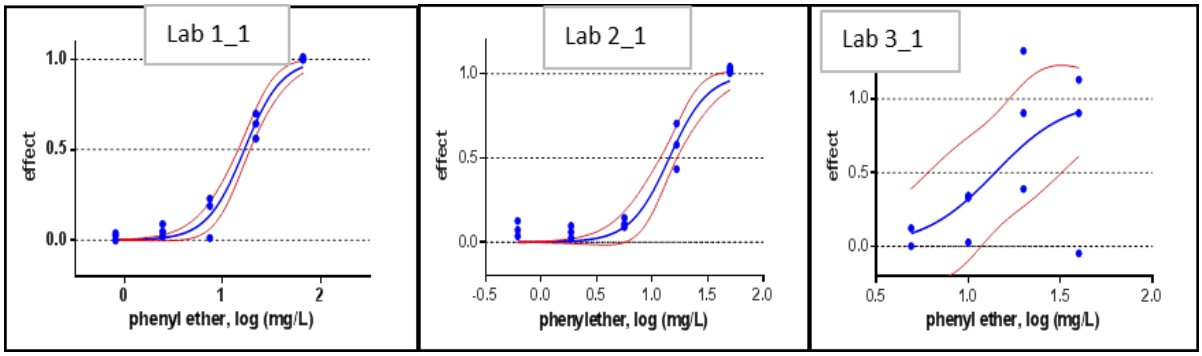
curve fit data: hexachlorophene

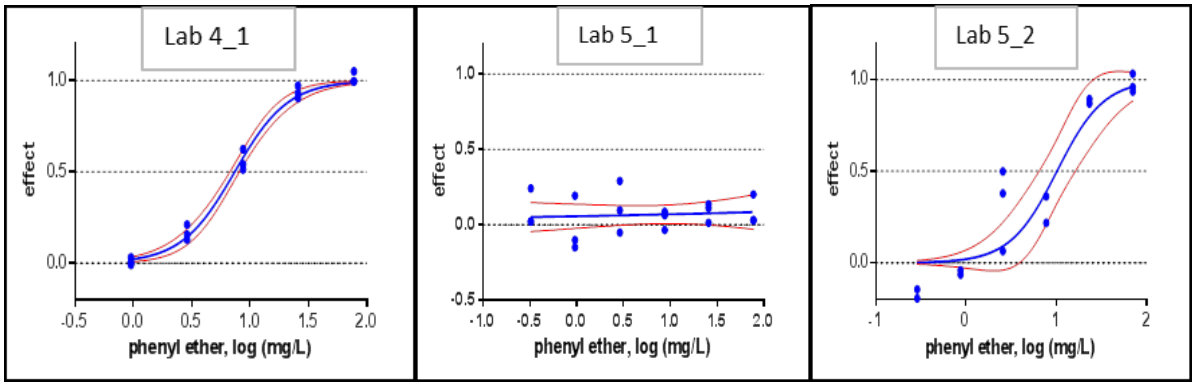
	Lab 1_1	Lab 2_1	Lab 3_1	Lab 4_1	Lab 5_2
type of fitting curve	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)
Best-fit values					
Top	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Bottom	= 0,0	= 0,0	= 0,0	= 0,0	= 0,0
LogIC50	0.07573	-0.0468	0.1128	0.08315	0.1269
HillSlope	1.973	1.45	1.926	1.611	2.215
IC50	1.19	0.8979	1.297	1.211	1.339
Span	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Std. Error					
LogIC50	0.04032	0.03791	0.01289	0.03215	0.07759
HillSlope	0.3438	0.1639	0.1099	0.1738	0.8719
95% Confidence Intervals					
LogIC50	-0,0114 to 0,1628	-0,1287 to 0,0351	0,0849 to 0,1406	0,0137 to 0,1526	-0,0376 to 0,2914
HillSlope	1,230 to 2,715	1,096 to 1,804	1,688 to 2,163	1,235 to 1,986	0,3662 to 4,063
IC50	0,9741 to 1,455	0,7435 to 1,084	1,216 to 1,382	1,032 to 1,421	0,9171 to 1,956
Goodness of Fit					
Degrees of Freedom	13	13	13	13	16
R square	0.9536	0.9644	0.9867	0.9752	0.8873
Absolute Sum of Squares	0.104	0.0651	0.01488	0.05277	0.5351
Sy.x	0.08944	0.07076	0.03383	0.06371	0.1829
Constraints					
Top	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000
Bottom	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0
Number of points					
Analyzed	15	15	15	15	18



curve fit data: phenyl ether

	Lab 1_1	Lab 2_1	Lab 3_1	Lab 4_1	Lab 4_2	Lab 5_1	Lab 5_2
type of fitting curve	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	Sigmoidal, 4PL, X is log(conc.)	no measurable effect	Sigmoidal, 4PL, X is log(conc.)
Best-fit values							
Top	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000
Bottom	= 0,0	= 0,0	= 0,0	= 0,0	= 0,0	= 0,0	= 0,0
LogIC50	1.222	1.154	1.145	0.8684	1.131	-	1.008
HillSlope	2.275	2.48	2.226	1.898	3.299	-	1.67
IC50	16.68	14.25	13.97	7.386	13.53	-	10.18
Span	= 1,000	= 1,000	= 1,000	= 1,000	= 1,000	-	= 1,000
Std. Error							
LogIC50	0.02734	0.03289	0.1384	0.01882	0.03184	-	0.08629
HillSlope	0.2891	0.4852	1.451	0.1431	0.7867	-	0.4899
95% Confidence Intervals							
LogIC50	1,163 to 1,281	1,083 to 1,225	0,8368 to 1,453	1,8277 to 0,9091	1,049 to 1,213	-	0,8250 to 1,191
HillSlope	1,651 to 2,900	1,431 to 3,528	-1,008 to 5,460	1,589 to 2,207	1,277 to 5,321	-	0,6315 to 2,708
IC50	14,56 to 19,11	12,10 to 16,79	6,867 to 28,40	6,726 to 8,111	11,20 to 16,33	-	6,683 to 15,52
Goodness of Fit							
Degrees of Freedom	13	13	10	13	5	-	16
R square	0.9768	0.9594	0.3597	0.9912	0.9822	-	0.8668
Absolute Sum of Squares	0.05292	0.08891	1.609	0.0214	0.02234	-	0.4769
Sy.x	0.0638	0.0827	0.4011	0.04057	0.06685	-	0.1726
Constraints							
Top	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000	Top = 1,000
Bottom	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0	Bottom = 0,0
Number of points							
Analyzed	15	15	12	15	7	18	18





7.4 Excel Spreadsheets with original data

Octylamine: laboratory 1. test run 1 (Lab_1_1)

AS sampling date	18-Apr-13
dry weight	2.6 g/L
test date	19-Apr-13
test substance	1-octylamine

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

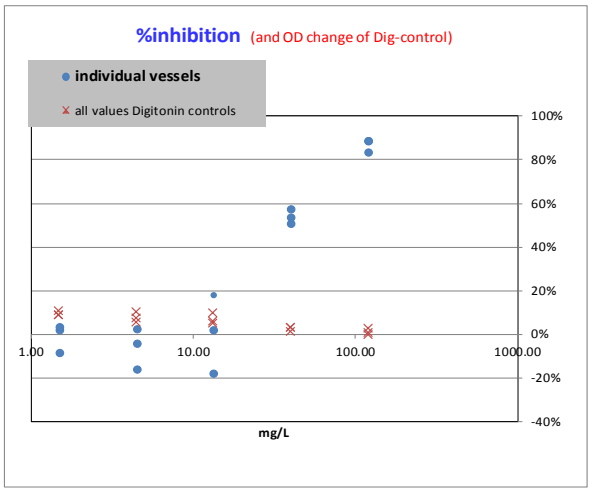
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1.26	0.44	0.82
$C0_b$	control	1.27	0.38	0.89
C1 in test	119.08	1.37	1.27	0.1
C2 in test	39.69	1.31	0.90	0.41
C3 in test	13.23	1.20	0.48	0.72
C4 in test	4.41	1.27	0.42	0.85
C5 in test	1.47	1.26	0.38	0.88
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1.25	0.35	0.9
$C0_b$	control	1.24	0.38	0.86
C1 in test	119.08	1.48	1.34	0.14
C2 in test	39.69	1.3	0.91	0.39
C3 in test	13.23	1.46	0.47	0.99
C4 in test	4.41	1.28	0.38	0.9
C5 in test	1.47	1.28	0.41	0.87
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1.30	0.40	0.9
$C0_b$	control	1.27	0.39	0.88
C1 in test	119.08	1.45	1.35	0.1
C2 in test	39.69	1.14	0.78	0.36
C3 in test	13.23	1.39	0.55	0.84
C4 in test	4.41	1.38	0.39	0.99
C5 in test	1.47	1.37	0.41	0.96
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1.45	1.34	0.11
$C0_b$+	control1	1.45	1.33	0.12
C1+ in test[1]	119.08	1.5	1.46	0.04
C2+ in test[1]	39.69	1.44	1.39	0.05
C3+ in test[1]	13.23	1.45	1.36	0.09
C4+ in test[1]	4.41	1.46	1.38	0.08
C5+ in test[1]	1.47	1.45	1.29	0.16
C6+ in test[1]				
C7+ in test[1]				

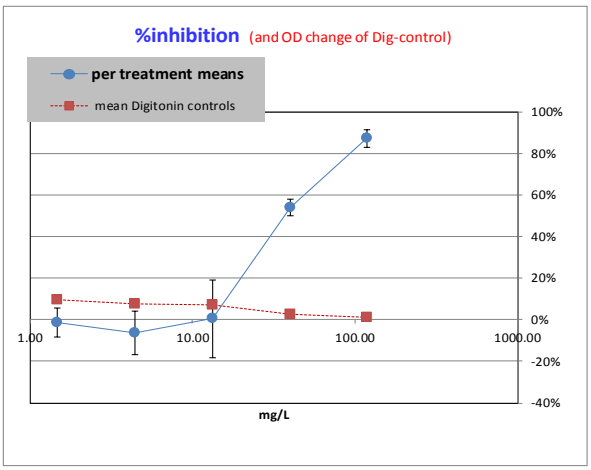
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1.45	1.35	0.1
$C0_b$+	control1	1.44	1.34	0.1
C1+ in test[1]	119.08	1.46	1.46	0
C2+ in test[1]	39.69	1.47	1.42	0.05
C3+ in test[1]	13.23	1.4	1.26	0.14
C4+ in test[1]	4.41	1.46	1.35	0.11
C5+ in test[1]	1.47	1.42	1.29	0.13
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1.52	1.40	0.12
$C0_b$+	control1	1.45	1.27	0.18
C1+ in test[1]	119.08	1.51	1.50	0.01
C2+ in test[1]	39.69	1.51	1.49	0.02
C3+ in test[1]	13.23	1.49	1.42	0.07
C4+ in test[1]	4.41	1.47	1.32	0.15
C5+ in test[1]	1.47	1.53	1.40	0.13
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



Octylamine: Lab_I_I (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,260	0,183	1,077	0,038	0,001	6	0,920	0,039	0%	4%	✓ 73%
mean C1	14,04	1,450	1,317	0,133	0,023	0,0005	3	0,070	0,024	92%	3%	5%
mean C2	4,68	1,353	0,720	0,633	0,031	0,0009	3	0,510	0,031	45%	3%	38%
mean C3	1,56	1,267	0,203	1,063	0,038	0,0014	3	0,917	0,041	0%	4%	72%
mean C4	0,52	1,223	0,177	1,047	0,064	0,0041	3	0,883	0,065	4%	7%	72%
mean C5	0,17	1,227	0,157	1,070	0,010	0,0001	3	0,920	0,010	0%	1%	75%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!
mean C7	#NV										0%	

Replicate2 1-3

mean $C0_0$+	0[1]	1,420	1,263	0,157	0,0103	0,0001	6					✓ 11%
mean C1+[1]	14,04	1,453	1,390	0,063	0,006	3,333E-05	3					✓ 4%
mean C2+[1]	4,68	1,453	1,330	0,123	0,006	3,333E-05	3					✓ 8%
mean C3+[1]	1,56	1,410	1,263	0,147	0,015	0,0002333	3					✓ 10%
mean C4+[1]	0,52	1,410	1,247	0,163	0,012	0,0001333	3					✓ 12%
mean C5+[1]	0,17	1,410	1,260	0,150	0,000	0	3					✓ 11%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

Octylamine: laboratory 2, test run 1 (Lab_2_1)

AS sampling date	14-Jan-13
dry weight	3,72 g/L
test date	15-Jan-13
test substance	1-Octylamin

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2	.	C2+
	C3	.	C3+
	C4	.	C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

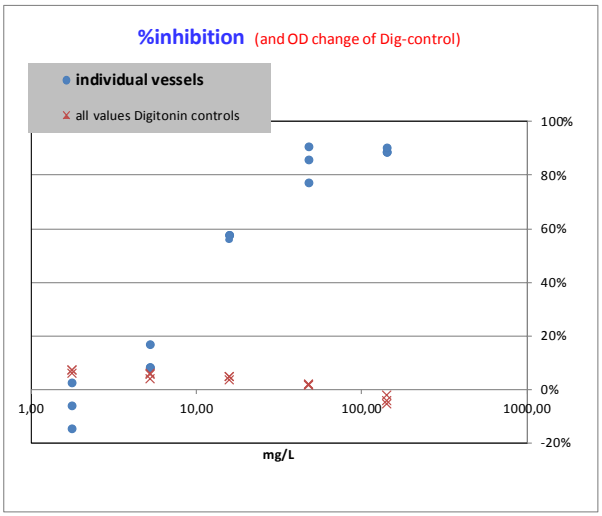
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,25	0,56	0,69
$C0_b$	control	1,38	0,72	0,66
C1 in test	142,24	1,51	1,50	0,01
C2 in test	47,41	1,37	1,29	0,08
C3 in test	15,80	1,42	1,10	0,32
C4 in test	5,27	1,33	0,72	0,61
C5 in test	1,76	1,33	0,61	0,72
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,36	0,66	0,7
$C0_b$	control	1,34	0,88	0,46
C1 in test	142,24	1,48	1,47	0,01
C2 in test	47,41	1,4	1,29	0,11
C3 in test	15,80	1,42	1,11	0,31
C4 in test	5,27	1,37	0,81	0,56
C5 in test	1,76	1,4	0,63	0,77
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,31	0,61	0,7
$C0_b$	control	1,39	0,70	0,69
C1 in test	142,24	1,50	1,50	0
C2 in test	47,41	1,47	1,31	0,16
C3 in test	15,80	1,41	1,10	0,31
C4 in test	5,27	1,38	0,77	0,61
C5 in test	1,76	1,36	0,69	0,67
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,50	1,36	0,14
$C0_b$+	control1	1,48	1,36	0,12
C1+ in test[1]	142,24	1,49	1,55	-0,06
C2+ in test[1]	47,41	1,46	1,43	0,03
C3+ in test[1]	15,8	1,49	1,42	0,07
C4+ in test[1]	5,27	1,46	1,4	0,06
C5+ in test[1]	1,76	1,49	1,38	0,11
C6+ in test[1]				
C7+ in test[1]				

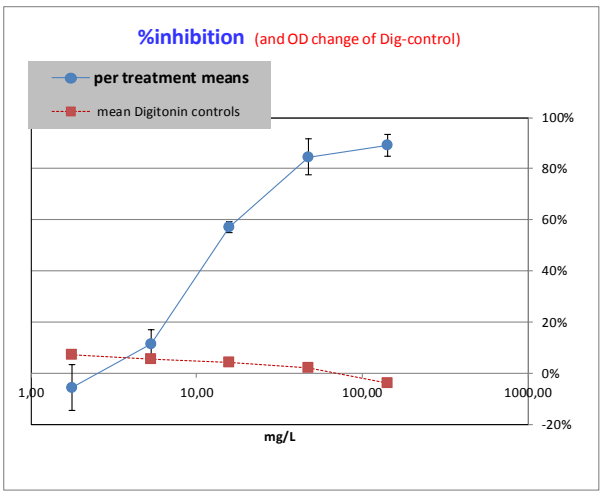
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,49	1,41	0,08
$C0_b$+	control1	1,49	1,41	0,08
C1+ in test[1]	142,24	1,49	1,52	-0,03
C2+ in test[1]	47,41	1,48	1,46	0,02
C3+ in test[1]	15,8	1,49	1,44	0,05
C4+ in test[1]	5,27	1,46	1,38	0,08
C5+ in test[1]	1,76	1,48	1,37	0,11
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,49	1,38	0,11
$C0_b$+	control1	1,52	1,41	0,11
C1+ in test[1]	142,24	1,51	1,59	-0,08
C2+ in test[1]	47,41	1,50	1,47	0,03
C3+ in test[1]	15,8	1,46	1,39	0,07
C4+ in test[1]	5,27	1,44	1,35	0,09
C5+ in test[1]	1,76	1,49	1,40	0,09
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



Octylamine: Lab_2_I (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,338	0,688	0,690	0,094	0,009	6	**0,583**	**0,097**	**0%**	17%	✓ 44%
	mean C1	142,24	1,497	1,490	0,007	0,006	0,0000	3	**0,063**	**0,026**	**89%**	4%	4%
	mean C2	47,41	1,413	1,297	0,117	0,040	0,0016	3	**0,090**	**0,041**	**85%**	7%	6%
	mean C3	15,80	1,417	1,103	0,313	0,006	0,0000	3	**0,250**	**0,013**	**57%**	2%	18%
	mean C4	5,27	1,360	0,767	0,593	0,029	0,0008	3	**0,517**	**0,033**	**11%**	6%	38%
	mean C5	1,76	1,363	0,643	0,720	0,050	0,0025	3	**0,617**	**0,051**	**-6%**	9%	45%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,495	1,388	0,107	0,0234	0,0005	6					✓ 7%
	mean C1+[1]	142,24	1,497	1,553	-0,057	0,025	0,0006333	3					✓ -4%
	mean C2+[1]	47,41	1,480	1,453	0,027	0,006	3,333E-05	3					✓ 2%
	mean C3+[1]	15,80	1,480	1,417	0,063	0,012	0,0001333	3					✓ 4%
	mean C4+[1]	5,27	1,453	1,377	0,077	0,015	0,0002333	3					✓ 5%
	mean C5+[1]	1,76	1,487	1,383	0,103	0,012	0,0001333	3					✓ 7%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

Octylamine: laboratory 3. test run 1 (Lab_3_1)

AS sampling date 12-Feb-13
dry weight 5,13 g/L

test date 12-Feb-13
test substance 1-octylamine

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

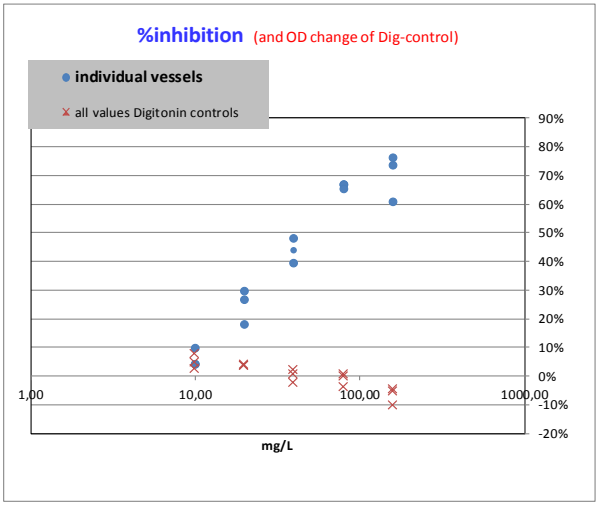
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	0,96	0,12	0,84
$C0_b$	control	0,98	0,13	0,85
C1 in test	157,01	1,17	0,98	0,19
C2 in test	78,51	1,13	0,91	0,22
C3 in test	39,25	1,07	0,67	0,4
C4 in test	19,63	1,11	0,48	0,63
C5 in test	9,81	1,03	0,28	0,75
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,05	0,18	0,87
$C0_b$	control	1,02	0,16	0,86
C1 in test	157,01	1,14	1,04	0,1
C2 in test	78,51	1,06	0,84	0,22
C3 in test	39,25	1,08	0,65	0,43
C4 in test	19,63	1,01	0,46	0,55
C5 in test	9,81	0,97	0,22	0,75
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	0,84	0,09	0,75
$C0_b$	control	0,89	0,09	0,8
C1 in test	157,01	1,12	1,04	0,08
C2 in test	78,51	1,08	0,85	0,23
C3 in test	39,25	1,05	0,68	0,37
C4 in test	19,63	1,04	0,47	0,57
C5 in test	9,81	1,04	0,33	0,71
C6 in test				
C7 in test				

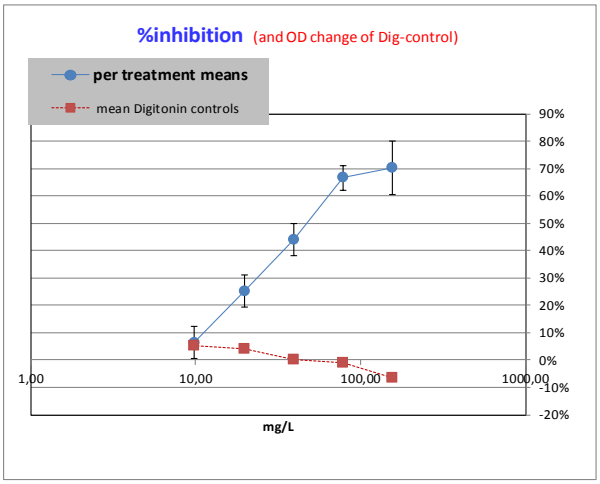


Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,33	1,20	0,13
$C0_b$+	control1	1,37	1,26	0,11
C1+ in test[1]	157,01	1,27	1,4	-0,13
C2+ in test[1]	78,51	1,39	1,39	0
C3+ in test[1]	39,25	1,37	1,34	0,03
C4+ in test[1]	19,63	1,4	1,35	0,05
C5+ in test[1]	9,81	1,35	1,31	0,04
C6+ in test[1]				
C7+ in test[1]				

Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,41	1,29	0,12
$C0_b$+	control1	1,37	1,28	0,09
C1+ in test[1]	157,01	1,4	1,46	-0,06
C2+ in test[1]	78,51	1,37	1,42	-0,05
C3+ in test[1]	39,25	1,34	1,33	0,01
C4+ in test[1]	19,63	1,37	1,31	0,06
C5+ in test[1]	9,81	1,39	1,32	0,07
C6+ in test[1]				
C7+ in test[1]				



Replicate 3 (+Digitonin)

$C0_a$+	control1	1,37	1,20	0,17
$C0_b$+	control1	1,31	1,21	0,1
C1+ in test[1]	157,01	1,29	1,36	-0,07
C2+ in test[1]	78,51	1,41	1,40	0,01
C3+ in test[1]	39,25	1,35	1,38	-0,03
C4+ in test[1]	19,63	1,33	1,28	0,05
C5+ in test[1]	9,81	1,45	1,34	0,11
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin

Octylamine: Lab_3_I (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	0,957	0,128	0,828	0,045	0,002	6	**0,708**	**0,053**	**0%**	8%	✓ 74%
	mean C1	157,01	1,143	1,020	0,123	0,059	0,0034	3	**0,210**	**0,070**	**70%**	10%	18%
	mean C2	78,51	1,090	0,867	0,223	0,006	0,0000	3	**0,237**	**0,033**	**67%**	5%	22%
	mean C3	39,25	1,067	0,667	0,400	0,030	0,0009	3	**0,397**	**0,043**	**44%**	6%	37%
	mean C4	19,63	1,053	0,470	0,583	0,042	0,0017	3	**0,530**	**0,042**	**25%**	6%	50%
	mean C5	9,81	1,013	0,277	0,737	0,023	0,0005	3	**0,663**	**0,042**	**6%**	6%	65%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,360	1,240	0,120	0,0283	0,0008	6					✓ 9%
	mean C1+[1]	157,01	1,320	1,407	-0,087	0,038	0,0014333	3					✗ -7%
	mean C2+[1]	78,51	1,390	1,403	-0,013	0,032	0,0010333	3					✓ -1%
	mean C3+[1]	39,25	1,353	1,350	0,003	0,031	0,0009333	3					✓ 0%
	mean C4+[1]	19,63	1,367	1,313	0,053	0,006	3,333E-05	3					✓ 4%
	mean C5+[1]	9,81	1,397	1,323	0,073	0,035	0,0012333	3					✓ 5%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

Octylamine: laboratory 4. test run 1 (Lab_4_1)

AS sampling date: 31-Aug-12
dry weight: 3,5 g/L

test date: 01-Sep-12
test substance: 1-octylamin

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

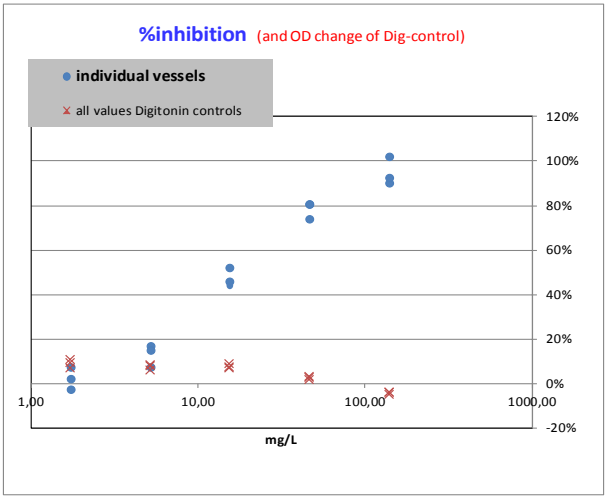
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,58	0,83	0,75
$C0_b$	control	1,60	0,86	0,74
C1 in test	138,86	1,64	1,64	-0,005
C2 in test	46,29	1,61	1,45	0,16
C3 in test	15,43	1,68	1,21	0,47
C4 in test	5,14	1,56	0,93	0,63
C5 in test	1,71	1,58	0,80	0,78
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,57	0,8	0,77
$C0_b$	control	1,59	0,83	0,76
C1 in test	138,86	1,61	1,69	-0,08
C2 in test	46,29	1,58	1,42	0,16
C3 in test	15,43	1,56	1,1	0,46
C4 in test	5,14	1,6	0,91	0,69
C5 in test	1,71	1,58	0,83	0,75
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,52	0,76	0,76
$C0_b$	control	1,56	0,79	0,77
C1 in test	138,86	1,59	1,61	-0,02
C2 in test	46,29	1,56	1,36	0,2
C3 in test	15,43	1,54	1,12	0,42
C4 in test	5,14	1,55	0,91	0,64
C5 in test	1,71	1,51	0,79	0,72
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,60	1,44	0,16
$C0_b$+	control1	1,63	1,51	0,12
C1+ in test[1]	138,86	1,65	1,73	-0,08
C2+ in test[1]	46,29	1,62	1,59	0,03
C3+ in test[1]	15,43	1,61	1,5	0,11
C4+ in test[1]	5,14	1,58	1,49	0,09
C5+ in test[1]	1,71	1,63	1,52	0,11
C6+ in test[1]				
C7+ in test[1]				

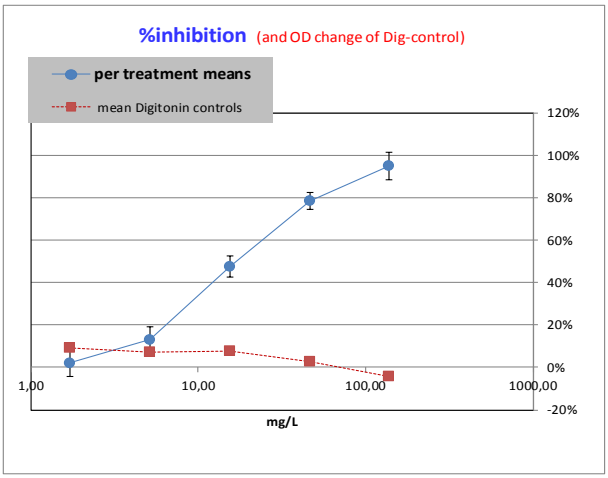
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,61	1,46	0,15
$C0_b$+	control1	1,59	1,47	0,12
C1+ in test[1]	138,86	1,59	1,65	-0,06
C2+ in test[1]	46,29	1,55	1,51	0,04
C3+ in test[1]	15,43	1,56	1,42	0,14
C4+ in test[1]	5,14	1,51	1,39	0,12
C5+ in test[1]	1,71	1,58	1,41	0,17
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,58	1,44	0,14
$C0_b$+	control1	1,62	1,49	0,13
C1+ in test[1]	138,86	1,59	1,65	-0,06
C2+ in test[1]	46,29	1,60	1,55	0,05
C3+ in test[1]	15,43	1,56	1,44	0,12
C4+ in test[1]	5,14	1,55	1,42	0,13
C5+ in test[1]	1,71	1,54	1,39	0,15
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



Octylamine: Lab_4_I (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,570	0,812	0,758	0,012	0,000	6	**0,622**	**0,020**	**0%**	3%	✓ 40%
mean C1	138,86	1,612	1,647	-0,035	0,040	0,0016	3	**0,032**	**0,041**	**95%**	7%	2%
mean C2	46,29	1,583	1,410	0,173	0,023	0,0005	3	**0,133**	**0,025**	**79%**	4%	8%
mean C3	15,43	1,593	1,143	0,450	0,026	0,0007	3	**0,327**	**0,031**	**47%**	5%	21%
mean C4	5,14	1,570	0,917	0,653	0,032	0,0010	3	**0,540**	**0,038**	**13%**	6%	34%
mean C5	1,71	1,557	0,807	0,750	0,030	0,0009	3	**0,607**	**0,043**	**2%**	7%	39%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
mean C7	#NV										**0%**	

Replicate2 1-3

mean $C0_b$+	0[1]	1,605	1,468	0,137	0,0163	0,0003	6					✓ 9%
mean C1+[1]	138,86	1,610	1,677	-0,067	0,012	0,0001333	3					✓ -4%
mean C2+[1]	46,29	1,590	1,550	0,040	0,010	0,0001	3					✓ 3%
mean C3+[1]	15,43	1,577	1,453	0,123	0,015	0,0002333	3					✓ 8%
mean C4+[1]	5,14	1,547	1,433	0,113	0,021	0,0004333	3					✓ 7%
mean C5+[1]	1,71	1,583	1,440	0,143	0,031	0,0009333	3					✓ 9%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

Octylamine: laboratory 5. test run 1 (Lab_5_1)

AS sampling date 17-Jul-12
dry weight 4,4 g/L

test date 18-Jul-12
test substance 1-octylamine

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

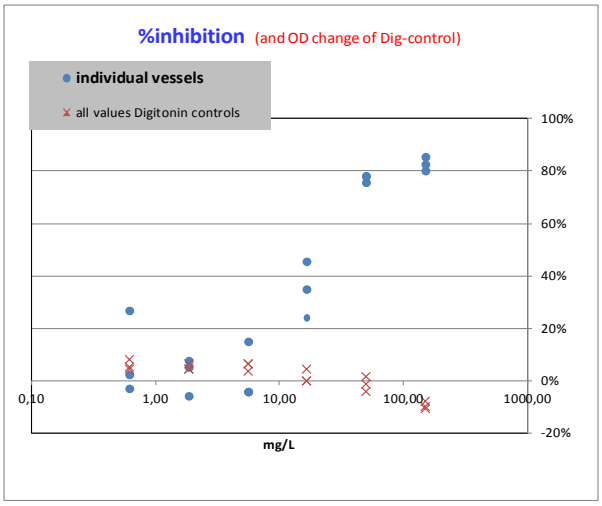
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,35	0,93	0,42
$C0_b$	control	1,36	0,94	0,42
C1 in test	150,55	1,35	1,43	-0,08
C2 in test	50,18	1,42	1,35	0,07
C3 in test	16,73	1,46	1,16	0,3
C4 in test	5,58	1,45	0,99	0,46
C5 in test	1,86	1,36	0,90	0,46
C6 in test	0,620	1,36	1,01	0,35
C7 in test				

Replicate 2

$C0_a$	control	1,36	0,9	0,46
$C0_b$	control	1,34	0,91	0,43
C1 in test	150,55	1,43	1,49	-0,06
C2 in test	50,18	1,38	1,32	0,06
C3 in test	16,73	1,41	1,15	0,26
C4 in test	5,58	1,39	1	0,39
C5 in test	1,86	1,42	1,01	0,41
C6 in test	0,62	1,27	0,81	0,46
C7 in test				

Replicate 3

$C0_a$	control	1,37	0,90	0,47
$C0_b$	control	1,35	0,98	0,37
C1 in test	150,55	1,38	1,45	-0,07
C2 in test	50,18	1,34	1,28	0,06
C3 in test	16,73	1,37	1,15	0,22
C4 in test	5,58	1,42	0,96	0,46
C5 in test	1,86	1,37	0,95	0,42
C6 in test	0,62	1,40	0,96	0,44
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,36	1,30	0,06
$C0_b$+	control1	1,35	1,31	0,04
C1+ in test[1]	150,55	1,42	1,53	-0,11
C2+ in test[1]	50,18	1,45	1,51	-0,06
C3+ in test[1]	16,73	1,42	1,42	0
C4+ in test[1]	5,58	1,4	1,35	0,05
C5+ in test[1]	1,86	1,41	1,32	0,09
C6+ in test[1]	0,62	1,36	1,30	0,06
C7+ in test[1]				

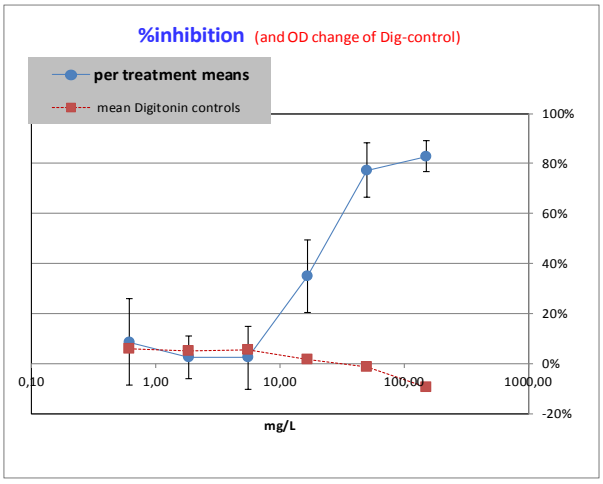
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,36	1,3	0,06
$C0_b$+	control1	1,38	1,31	0,07
C1+ in test[1]	150,55	1,38	1,52	-0,14
C2+ in test[1]	50,18	1,39	1,41	-0,02
C3+ in test[1]	16,73	1,45	1,39	0,06
C4+ in test[1]	5,58	1,41	1,32	0,09
C5+ in test[1]	1,86	1,4	1,34	0,06
C6+ in test[1]	0,62	1,41	1,30	0,11
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,37	1,31	0,06
$C0_b$+	control1	1,36	1,29	0,07
C1+ in test[1]	150,55	1,38	1,53	-0,15
C2+ in test[1]	50,18	1,38	1,36	0,02
C3+ in test[1]	16,73	1,38	1,38	0
C4+ in test[1]	5,58	1,39	1,30	0,09
C5+ in test[1]	1,86	1,36	1,30	0,06
C6+ in test[1]	0,62	1,40	1,33	0,07
C7+ in test[1]				

[1]) including Digitonin



Octylamine: Lab_5_I (continued)

data analysis / evaluation

	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,355	0,927	0,428	0,035	0,001	6	**0,368**	**0,037**	**0%**	10%	✓ 27%
	mean C1	150,55	1,387	1,457	-0,070	0,010	0,0001	3	**0,063**	**0,023**	**83%**	6%	5%
	mean C2	50,18	1,380	1,317	0,063	0,006	0,0000	3	**0,083**	**0,040**	**77%**	11%	6%
	mean C3	16,73	1,413	1,153	0,260	0,040	0,0016	3	**0,240**	**0,053**	**35%**	14%	17%
	mean C4	5,58	1,420	0,983	0,437	0,040	0,0016	3	**0,360**	**0,047**	**2%**	13%	25%
	mean C5	1,86	1,383	0,953	0,430	0,026	0,0007	3	**0,360**	**0,032**	**2%**	9%	26%
	mean C6	0,620	1,343	0,927	0,417	0,059	0,0034	3	**0,337**	**0,064**	**9%**	17%	25%
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,363	1,303	0,060	0,0110	0,0001	6					✓ 4%
	mean C1+[1]	150,55	1,393	1,527	-0,133	0,021	0,0004333	3					✗ -10%
	mean C2+[1]	50,18	1,407	1,427	-0,020	0,040	0,0016	3					✓ -1%
	mean C3+[1]	16,73	1,417	1,397	0,020	0,035	0,0012	3					✓ 1%
	mean C4+[1]	5,58	1,400	1,323	0,077	0,023	0,0005333	3					✓ 5%
	mean C5+[1]	1,86	1,390	1,320	0,070	0,017	0,0003	3					✓ 5%
	mean C6+[1]	0,62	1,390	1,310	0,080	0,026	0,0007	3					✓ 6%
	mean C7+[1]	#NV											

[1]) including Digitonin

Octylamine: laboratory 5. test run 2 (Lab_5_2)

AS sampling date: 31-Jul-12
dry weight: 4,9 g/L

test date: 02-Aug-12
test substance: 1-octylamine

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

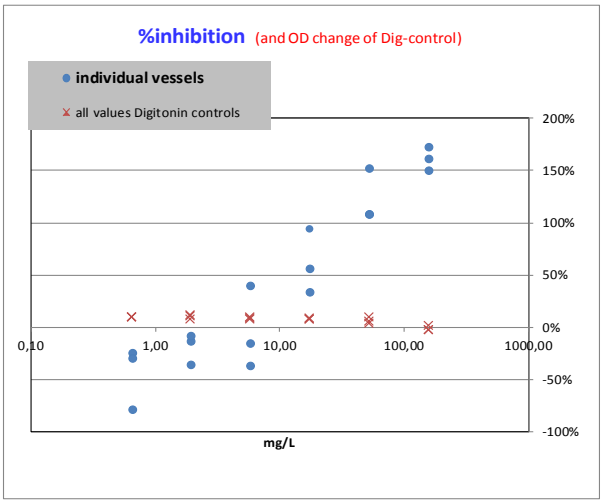
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,37	1,01	0,36
$C0_b$	control	1,34	1,05	0,29
C1 in test	155,18	1,46	1,61	-0,15
C2 in test	51,73	1,49	1,41	0,08
C3 in test	17,24	1,37	1,24	0,13
C4 in test	5,75	1,33	1,09	0,24
C5 in test	1,92	1,39	1,03	0,36
C6 in test	0,640	1,44	0,97	0,47
C7 in test				

Replicate 2

$C0_a$	control	1,35	1,02	0,33
$C0_b$	control	1,33	0,95	0,38
C1 in test	155,18	1,48	1,59	-0,11
C2 in test	51,73	1,4	1,4	0
C3 in test	17,24	1,47	1,23	0,24
C4 in test	5,75	1,37	1,03	0,34
C5 in test	1,92	1,38	1,03	0,35
C6 in test	0,64	1,34	0,97	0,37
C7 in test				

Replicate 3

$C0_a$	control	1,30	0,95	0,35
$C0_b$	control	1,35	0,97	0,38
C1 in test	155,18	1,48	1,61	-0,13
C2 in test	51,73	1,47	1,39	0,08
C3 in test	17,24	1,35	1,15	0,2
C4 in test	5,75	1,36	0,98	0,38
C5 in test	1,92	1,33	0,93	0,4
C6 in test	0,64	1,35	0,97	0,38
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,48	1,29	0,19
$C0_b$+	control1	1,52	1,34	0,18
C1+ in test[1]	155,18	1,61	1,64	-0,03
C2+ in test[1]	51,73	1,52	1,37	0,15
C3+ in test[1]	17,24	1,47	1,36	0,11
C4+ in test[1]	5,75	1,49	1,35	0,14
C5+ in test[1]	1,92	1,49	1,31	0,18
C6+ in test[1]	0,64	1,49	1,35	0,14
C7+ in test[1]				

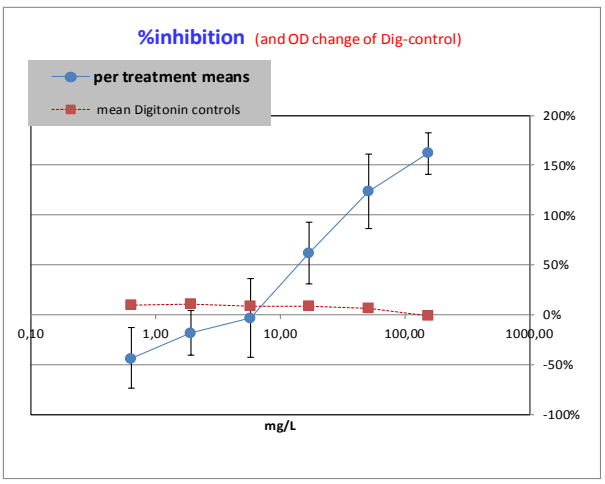
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,49	1,33	0,16
$C0_b$+	control1	1,46	1,31	0,15
C1+ in test[1]	155,18	1,56	1,54	0,02
C2+ in test[1]	51,73	1,51	1,42	0,09
C3+ in test[1]	17,24	1,49	1,37	0,12
C4+ in test[1]	5,75	1,48	1,36	0,12
C5+ in test[1]	1,92	1,5	1,34	0,16
C6+ in test[1]	0,64	1,46	1,32	0,14
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,45	1,29	0,16
$C0_b$+	control1	1,48	1,33	0,15
C1+ in test[1]	155,18	1,52	1,56	-0,04
C2+ in test[1]	51,73	1,48	1,43	0,05
C3+ in test[1]	17,24	1,48	1,35	0,13
C4+ in test[1]	5,75	1,47	1,34	0,13
C5+ in test[1]	1,92	1,48	1,36	0,12
C6+ in test[1]	0,64	1,48	1,33	0,15
C7+ in test[1]				

[1]) including Digitonin



Octylamine: Lab_5_2 (continued)

	data analysis / evaluation											
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,340	0,992	0,348	0,034	0,001	6	**0,183**	**0,038**	**0%**	21%	✗ 14%
	mean C1	155,18	1,473	1,603	-0,130	0,020	0,0004	3	**-0,113**	**0,038**	**162%**	21%	-8%
	mean C2	51,73	1,453	1,400	0,053	0,046	0,0021	3	**-0,043**	**0,068**	**124%**	37%	-3%
	mean C3	17,24	1,397	1,207	0,190	0,056	0,0031	3	**0,070**	**0,057**	**62%**	31%	5%
	mean C4	5,75	1,353	1,033	0,320	0,072	0,0052	3	**0,190**	**0,073**	**-4%**	40%	14%
	mean C5	1,92	1,367	0,997	0,370	0,026	0,0007	3	**0,217**	**0,040**	**-18%**	22%	16%
	mean C6	0,640	1,377	0,970	0,407	0,055	0,0030	3	**0,263**	**0,055**	**-44%**	30%	19%
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,480	1,315	0,165	0,0164	0,0003	6					✓ 11%
	mean C1+[1]	155,18	1,563	1,580	-0,017	0,032	0,0010333	3					✓ -1%
	mean C2+[1]	51,73	1,503	1,407	0,097	0,050	0,0025333	3					✓ 6%
	mean C3+[1]	17,24	1,480	1,360	0,120	0,010	0,0001	3					✓ 8%
	mean C4+[1]	5,75	1,480	1,350	0,130	0,010	0,0001	3					✓ 9%
	mean C5+[1]	1,92	1,490	1,337	0,153	0,031	0,0009333	3					✓ 10%
	mean C6+[1]	0,64	1,477	1,333	0,143	0,006	3,33333E-05	3					✓ 10%
	mean C7+[1]	#NV											

[1]) including Digitonin

3.5-dichlorophenol: laboratory 1. test run 1 (Lab_1_1)

AS sampling date 28-Feb-13
dry weight 3,6 g/L

test date 28-Feb-13
test substance 3,5-DCP

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

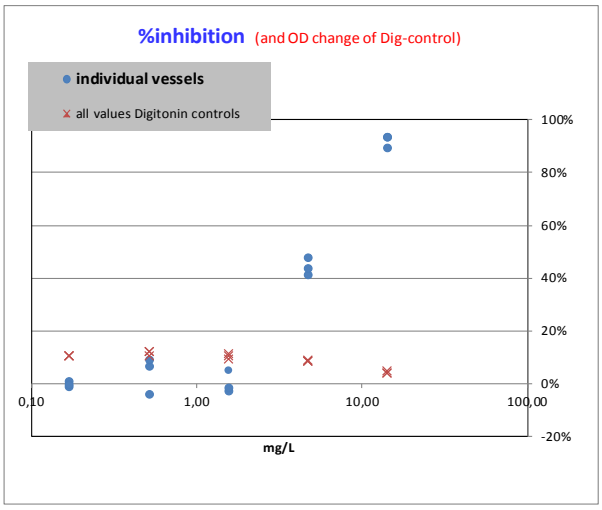
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,31	0,22	1,09
$C0_b$	control	1,24	0,21	1,03
C1 in test	14,04	1,46	1,34	0,12
C2 in test	4,68	1,36	0,76	0,6
C3 in test	1,56	1,23	0,21	1,02
C4 in test	0,52	1,23	0,21	1,02
C5 in test	0,17	1,22	0,16	1,06
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,25	0,18	1,07
$C0_b$	control	1,26	0,22	1,04
C1 in test	14,04	1,39	1,27	0,12
C2 in test	4,68	1,33	0,67	0,66
C3 in test	1,56	1,29	0,2	1,09
C4 in test	0,52	1,31	0,19	1,12
C5 in test	0,17	1,25	0,17	1,08
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,25	0,15	1,1
$C0_b$	control	1,25	0,12	1,13
C1 in test	14,04	1,50	1,34	0,16
C2 in test	4,68	1,37	0,73	0,64
C3 in test	1,56	1,28	0,20	1,08
C4 in test	0,52	1,13	0,13	1
C5 in test	0,17	1,21	0,14	1,07
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,42	1,27	0,15
$C0_b$+	control1	1,40	1,26	0,14
C1+ in test[1]	14,04	1,46	1,4	0,06
C2+ in test[1]	4,68	1,56	1,44	0,12
C3+ in test[1]	1,56	1,42	1,29	0,13
C4+ in test[1]	0,52	1,4	1,23	0,17
C5+ in test[1]	0,17	1,44	1,29	0,15
C6+ in test[1]				
C7+ in test[1]				

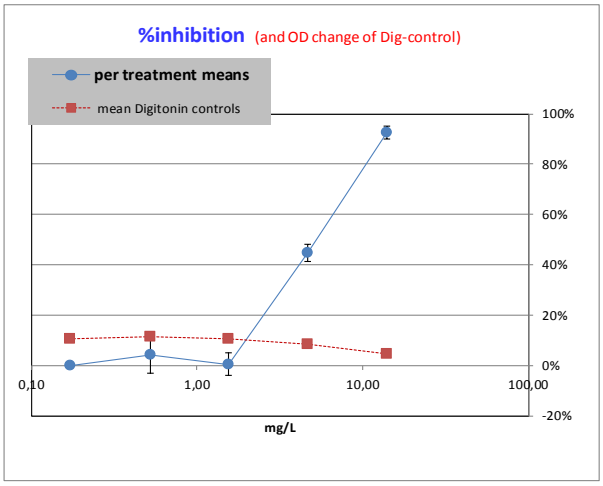
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,44	1,28	0,16
$C0_b$+	control1	1,43	1,27	0,16
C1+ in test[1]	14,04	1,45	1,38	0,07
C2+ in test[1]	4,68	1,36	1,23	0,13
C3+ in test[1]	1,56	1,38	1,22	0,16
C4+ in test[1]	0,52	1,41	1,26	0,15
C5+ in test[1]	0,17	1,41	1,26	0,15
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,42	1,25	0,17
$C0_b$+	control1	1,41	1,25	0,16
C1+ in test[1]	14,04	1,45	1,39	0,06
C2+ in test[1]	4,68	1,44	1,32	0,12
C3+ in test[1]	1,56	1,43	1,28	0,15
C4+ in test[1]	0,52	1,42	1,25	0,17
C5+ in test[1]	0,17	1,38	1,23	0,15
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



3.5-dichlorophenol: Lab_I_I (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
mean C0	0	1,260	0,183	1,077	0,038	0,001	6	**0,920**	**0,039**	**0%**	4%	✓ 73%
mean C1	14,04	1,450	1,317	0,133	0,023	0,0005	3	**0,070**	**0,024**	**92%**	3%	5%
mean C2	4,68	1,353	0,720	0,633	0,031	0,0009	3	**0,510**	**0,031**	**45%**	3%	38%
mean C3	1,56	1,267	0,203	1,063	0,038	0,0014	3	**0,917**	**0,041**	**0%**	4%	72%
mean C4	0,52	1,223	0,177	1,047	0,064	0,0041	3	**0,883**	**0,065**	**4%**	7%	72%
mean C5	0,17	1,227	0,157	1,070	0,010	0,0001	3	**0,920**	**0,010**	**0%**	1%	75%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
mean C7	#NV										0%	

Replicate2 1-3

mean $C0_b$+	0[1]	1,420	1,263	0,157	0,0103	0,0001	6					✓ 11%
mean C1+[1]	14,04	1,453	1,390	0,063	0,006	3,333E-05	3					✓ 4%
mean C2+[1]	4,68	1,453	1,330	0,123	0,006	3,333E-05	3					✓ 8%
mean C3+[1]	1,56	1,410	1,263	0,147	0,015	0,0002333	3					✓ 10%
mean C4+[1]	0,52	1,410	1,247	0,163	0,012	0,0001333	3					✓ 12%
mean C5+[1]	0,17	1,410	1,260	0,150	0,000	0	3					✓ 11%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

3.5-dichlorophenol: laboratory 2. test run 1 (Lab_2_1)

AS sampling date: 14-Jan-13
dry weight: 3,72 g/L

test date: 15-Jan-13
test substance: 3,5-Dichlorophenol

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

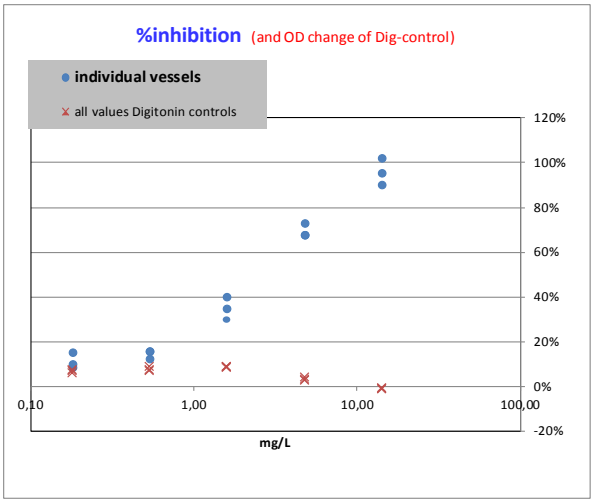
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,34	0,68	0,66
$C0_b$	control	1,33	0,61	0,72
C1 in test	14,22	1,49	1,45	0,04
C2 in test	4,74	1,43	1,19	0,24
C3 in test	1,58	1,44	0,90	0,54
C4 in test	0,53	1,37	0,76	0,61
C5 in test	0,18	1,38	0,78	0,6
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,33	0,65	0,68
$C0_b$	control	1,33	0,65	0,68
C1 in test	14,22	1,42	1,45	-0,03
C2 in test	4,74	1,42	1,21	0,21
C3 in test	1,58	1,39	0,88	0,51
C4 in test	0,53	1,36	0,73	0,63
C5 in test	0,18	1,42	0,78	0,64
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,36	0,66	0,7
$C0_b$	control	1,36	0,64	0,72
C1 in test	14,22	1,45	1,44	0,01
C2 in test	4,74	1,48	1,24	0,24
C3 in test	1,58	1,38	0,90	0,48
C4 in test	0,53	1,39	0,78	0,61
C5 in test	0,18	1,39	0,76	0,63
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,46	1,37	0,09
$C0_b$+	control1	1,48	1,40	0,08
C1+ in test[1]	14,22	1,51	1,53	-0,02
C2+ in test[1]	4,74	1,5	1,44	0,06
C3+ in test[1]	1,58	1,48	1,35	0,13
C4+ in test[1]	0,53	1,44	1,33	0,11
C5+ in test[1]	0,18	1,48	1,38	0,1
C6+ in test[1]				
C7+ in test[1]				

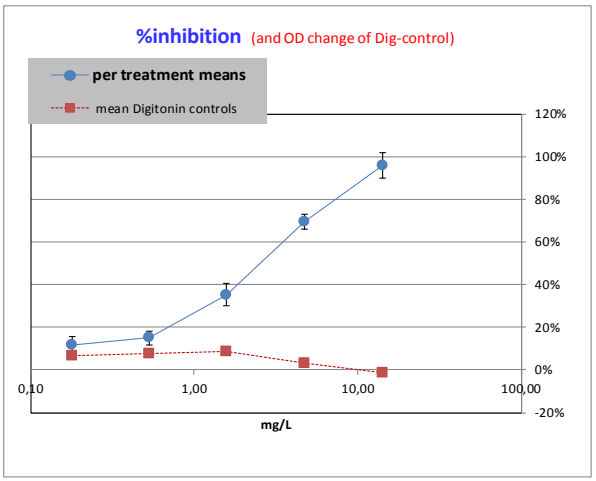
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,42	1,31	0,11
$C0_b$+	control1	1,51	1,38	0,13
C1+ in test[1]	14,22	1,55	1,56	-0,01
C2+ in test[1]	4,74	1,49	1,44	0,05
C3+ in test[1]	1,58	1,45	1,33	0,12
C4+ in test[1]	0,53	1,45	1,35	0,1
C5+ in test[1]	0,18	1,41	1,3	0,11
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,40	1,30	0,1
$C0_b$+	control1	1,47	1,37	0,1
C1+ in test[1]	14,22	1,51	1,53	-0,02
C2+ in test[1]	4,74	1,43	1,39	0,04
C3+ in test[1]	1,58	1,44	1,31	0,13
C4+ in test[1]	0,53	1,44	1,31	0,13
C5+ in test[1]	0,18	1,45	1,36	0,09
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



3.5-dichlorophenol: Lab_2_1 (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,342	0,648	0,693	0,024	0,001	6	**0,592**	**0,030**	**0%**	5%	✓ 44%
	mean C1	14,22	1,453	1,447	0,007	0,035	0,0012	3	**0,023**	**0,036**	**96%**	6%	2%
	mean C2	4,74	1,443	1,213	0,230	0,017	0,0003	3	**0,180**	**0,020**	**70%**	3%	12%
	mean C3	1,58	1,403	0,893	0,510	0,030	0,0009	3	**0,383**	**0,031**	**35%**	5%	27%
	mean C4	0,53	1,373	0,757	0,617	0,012	0,0001	3	**0,503**	**0,019**	**15%**	3%	37%
	mean C5	0,18	1,397	0,773	0,623	0,021	0,0004	3	**0,523**	**0,023**	**12%**	4%	37%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,457	1,355	0,102	0,0172	0,0003	6					✓ 7%
	mean C1+[1]	14,22	1,523	1,540	-0,017	0,006	3,333E-05	3					✓ -1%
	mean C2+[1]	4,74	1,473	1,423	0,050	0,010	0,0001	3					✓ 3%
	mean C3+[1]	1,58	1,457	1,330	0,127	0,006	3,333E-05	3					✓ 9%
	mean C4+[1]	0,53	1,443	1,330	0,113	0,015	0,0002333	3					✓ 8%
	mean C5+[1]	0,18	1,447	1,347	0,100	0,010	0,0001	3					✓ 7%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

3.5-dichlorophenol: laboratory 3. test run 1 (Lab_3_1)

AS sampling date	12-Feb-13
dry weight	5,13 g/L
test date	12-Feb-13
test substance	3,5-dichlorophenol

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

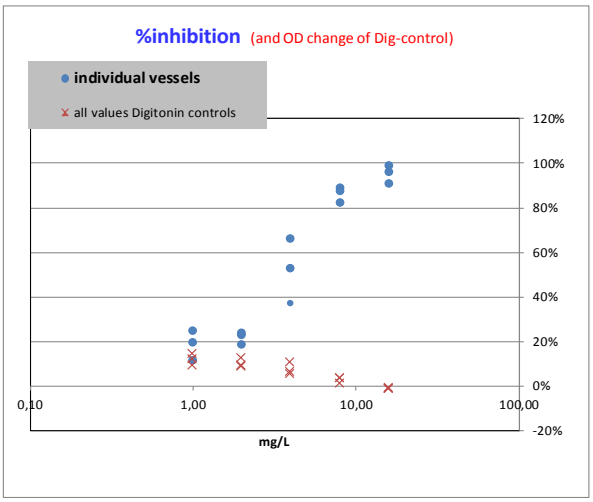
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,22	0,29	0,93
$C0_b$	control	1,24	0,36	0,88
C1 in test	15,70	1,47	1,42	0,05
C2 in test	7,85	1,45	1,28	0,17
C3 in test	3,93	1,44	0,86	0,58
C4 in test	1,96	1,35	0,63	0,72
C5 in test	0,98	1,26	0,43	0,83
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,34	0,42	0,92
$C0_b$	control	1,27	0,42	0,85
C1 in test	15,70	1,51	1,5	0,01
C2 in test	7,85	1,48	1,36	0,12
C3 in test	3,93	1,39	0,93	0,46
C4 in test	1,96	1,31	0,6	0,71
C5 in test	0,98	0,91	0,14	0,77
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,31	0,46	0,85
$C0_b$	control	1,26	0,35	0,91
C1 in test	15,70	1,44	1,45	-0,01
C2 in test	7,85	1,43	1,30	0,13
C3 in test	3,93	1,48	1,12	0,36
C4 in test	1,96	1,31	0,56	0,75
C5 in test	0,98	1,06	0,33	0,73
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,32	1,20	0,12
$C0_b$+	control1	1,39	1,26	0,13
C1+ in test[1]	15,70	1,71	1,73	-0,02
C2+ in test[1]	7,85	1,4	1,35	0,05
C3+ in test[1]	3,93	1,34	1,25	0,09
C4+ in test[1]	1,96	1,34	1,22	0,12
C5+ in test[1]	0,98	1,35	1,22	0,13
C6+ in test[1]				
C7+ in test[1]				

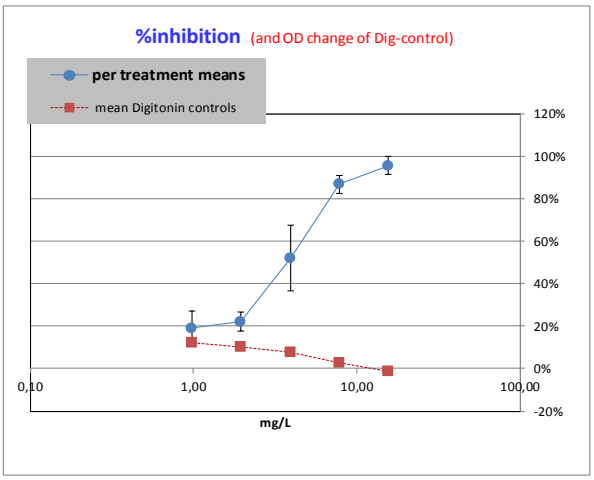
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,35	1,18	0,17
$C0_b$+	control1	1,34	1,21	0,13
C1+ in test[1]	15,70	1,43	1,44	-0,01
C2+ in test[1]	7,85	1,39	1,37	0,02
C3+ in test[1]	3,93	1,44	1,36	0,08
C4+ in test[1]	1,96	1,36	1,19	0,17
C5+ in test[1]	0,98	1,41	1,24	0,17
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,31	1,18	0,13
$C0_b$+	control1	1,34	1,20	0,14
C1+ in test[1]	15,70	1,42	1,44	-0,02
C2+ in test[1]	7,85	1,36	1,31	0,05
C3+ in test[1]	3,93	1,44	1,29	0,15
C4+ in test[1]	1,96	1,38	1,25	0,13
C5+ in test[1]	0,98	1,38	1,18	0,2
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



3.5-dichlorophenol: Lab_3_1 (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,273	0,383	0,890	0,035	0,001	6	**0,753**	**0,039**	**0%**	5%	✓ 59%
	mean C1	15,70	1,473	1,457	0,017	0,031	0,0009	3	**0,033**	**0,031**	**96%**	4%	2%
	mean C2	7,85	1,453	1,313	0,140	0,026	0,0007	3	**0,100**	**0,032**	**87%**	4%	7%
	mean C3	3,93	1,437	0,970	0,467	0,110	0,0121	3	**0,360**	**0,116**	**52%**	15%	25%
	mean C4	1,96	1,323	0,597	0,727	0,021	0,0004	3	**0,587**	**0,034**	**22%**	4%	44%
	mean C5	0,98	1,077	0,300	0,777	0,050	0,0025	3	**0,610**	**0,061**	**19%**	8%	57%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,342	1,205	0,137	0,0175	0,0003	6					✓ 10%
	mean C1+[1]	15,70	1,520	1,537	-0,017	0,006	3,333E-05	3					✓ -1%
	mean C2+[1]	7,85	1,383	1,343	0,040	0,017	0,0003	3					✓ 3%
	mean C3+[1]	3,93	1,407	1,300	0,107	0,038	0,0014333	3					✓ 8%
	mean C4+[1]	1,96	1,360	1,220	0,140	0,026	0,0007	3					✓ 10%
	mean C5+[1]	0,98	1,380	1,213	0,167	0,035	0,0012333	3					✓ 12%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

3.5-dichlorophenol: laboratory 4. test run 1 (Lab_4_1)

AS sampling date: 31-Aug-12
dry weight: 3,5 g/L

test date: 01-Sep-12
test substance: 3,5-dichlorophenol

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

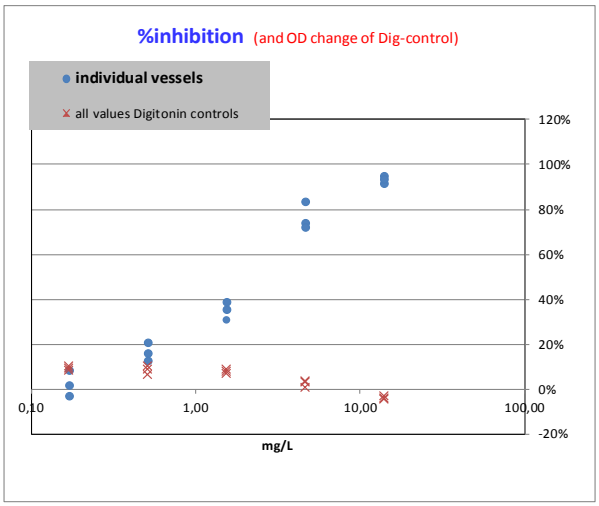
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,54	0,78	0,76
$C0_b$	control	1,58	0,84	0,74
C1 in test	13,89	1,59	1,60	-0,01
C2 in test	4,63	1,58	1,44	0,14
C3 in test	1,54	1,55	1,00	0,55
C4 in test	0,51	1,56	0,89	0,67
C5 in test	0,17	1,54	0,76	0,78
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,55	0,79	0,76
$C0_b$	control	1,56	0,81	0,75
C1 in test	13,89	1,56	1,59	-0,03
C2 in test	4,63	1,57	1,37	0,2
C3 in test	1,54	1,53	1,01	0,52
C4 in test	0,51	1,54	0,92	0,62
C5 in test	0,17	1,56	0,81	0,75
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,53	0,78	0,75
$C0_b$	control	1,54	0,77	0,77
C1 in test	13,89	1,56	1,58	-0,02
C2 in test	4,63	1,54	1,33	0,21
C3 in test	1,54	1,52	1,02	0,5
C4 in test	0,51	1,56	0,91	0,65
C5 in test	0,17	1,52	0,81	0,71
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,58	1,44	0,14
$C0_b$+	control1	1,56	1,43	0,13
C1+ in test[1]	13,89	1,6	1,65	-0,05
C2+ in test[1]	4,63	1,58	1,57	0,01
C3+ in test[1]	1,54	1,54	1,43	0,11
C4+ in test[1]	0,51	1,55	1,45	0,1
C5+ in test[1]	0,17	1,55	1,42	0,13
C6+ in test[1]				
C7+ in test[1]				

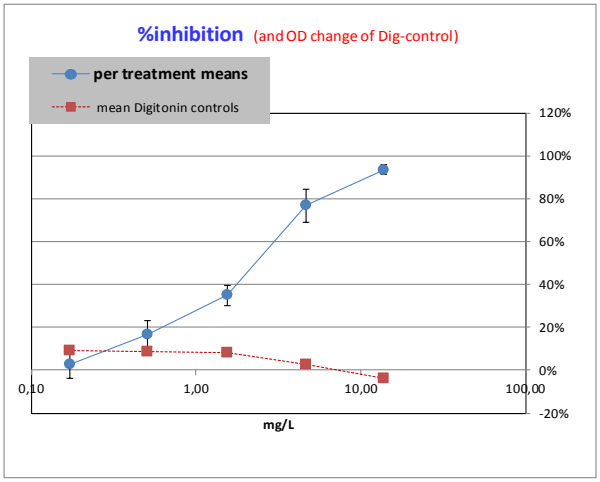
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,58	1,43	0,15
$C0_b$+	control1	1,57	1,43	0,14
C1+ in test[1]	13,89	1,58	1,64	-0,06
C2+ in test[1]	4,63	1,56	1,5	0,06
C3+ in test[1]	1,54	1,53	1,41	0,12
C4+ in test[1]	0,51	1,54	1,38	0,16
C5+ in test[1]	0,17	1,57	1,41	0,16
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,55	1,42	0,13
$C0_b$+	control1	1,59	1,44	0,15
C1+ in test[1]	13,89	1,56	1,63	-0,07
C2+ in test[1]	4,63	1,56	1,51	0,05
C3+ in test[1]	1,54	1,54	1,40	0,14
C4+ in test[1]	0,51	1,53	1,39	0,14
C5+ in test[1]	0,17	1,56	1,41	0,15
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



3.5-dichlorophenol: Lab_4_1 (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,550	0,795	0,755	0,010	0,000	6	**0,615**	**0,014**	**0%**	2%	✓ 40%
mean C1	13,89	1,570	1,590	-0,020	0,010	0,0001	3	**0,040**	**0,014**	**93%**	2%	3%
mean C2	4,63	1,563	1,380	0,183	0,038	0,0014	3	**0,143**	**0,046**	**77%**	8%	9%
mean C3	1,54	1,533	1,010	0,523	0,025	0,0006	3	**0,400**	**0,029**	**35%**	5%	26%
mean C4	0,51	1,553	0,907	0,647	0,025	0,0006	3	**0,513**	**0,040**	**17%**	6%	33%
mean C5	0,17	1,540	0,793	0,747	0,035	0,0012	3	**0,600**	**0,038**	**2%**	6%	39%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
mean C7	#NV										0%	

Replicate2 1-3

mean $C0_b$+	0[1]	1,572	1,432	0,140	0,0089	0,0001	6					✓ 9%
mean C1+[1]	13,89	1,580	1,640	-0,060	0,010	0,0001	3					✓ -4%
mean C2+[1]	4,63	1,567	1,527	0,040	0,026	0,0007	3					✓ 3%
mean C3+[1]	1,54	1,537	1,413	0,123	0,015	0,0002333	3					✓ 8%
mean C4+[1]	0,51	1,540	1,407	0,133	0,031	0,0009333	3					✓ 9%
mean C5+[1]	0,17	1,560	1,413	0,147	0,015	0,0002333	3					✓ 9%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

3.5-dichlorophenol: laboratory 5. test run 1 (Lab_5_1)

AS sampling date: 11-Jul-12
dry weight: 5,2 g/L

test date: 12-Jul-12
test substance: 3,5-Dichlorophenol

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

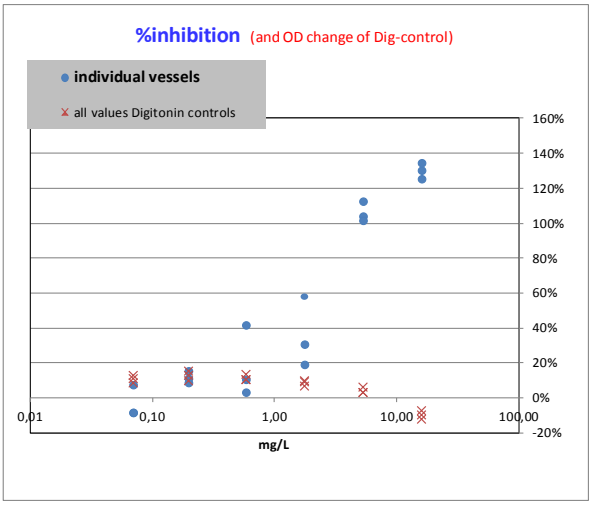
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,33	0,80	0,53
$C0_b$	control	1,36	0,80	0,56
C1 in test	15,91	1,40	1,67	-0,27
C2 in test	5,30	1,42	1,42	0
C3 in test	1,77	1,41	1,10	0,31
C4 in test	0,59	1,40	0,98	0,42
C5 in test	0,20	1,43	0,85	0,58
C6 in test	0,070	1,41	0,85	0,56
C7 in test				

Replicate 2

$C0_a$	control	1,41	0,81	0,6
$C0_b$	control	1,32	0,71	0,61
C1 in test	15,91	1,43	1,68	-0,25
C2 in test	5,30	1,44	1,4	0,04
C3 in test	1,77	1,41	0,98	0,43
C4 in test	0,59	1,39	0,8	0,59
C5 in test	0,20	1,4	0,85	0,55
C6 in test	0,07	1,43	0,8	0,63
C7 in test				

Replicate 3

$C0_a$	control	1,35	0,74	0,61
$C0_b$	control	1,35	0,75	0,6
C1 in test	15,91	1,43	1,72	-0,29
C2 in test	5,30	1,42	1,37	0,05
C3 in test	1,77	1,44	0,96	0,48
C4 in test	0,59	1,40	0,84	0,56
C5 in test	0,20	1,37	0,80	0,57
C6 in test	0,07	1,44	0,81	0,63
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,39	1,25	0,14
$C0_b$+	control1	1,43	1,28	0,15
C1+ in test[1]	15,91	1,46	1,56	-0,1
C2+ in test[1]	5,3	1,44	1,4	0,04
C3+ in test[1]	1,77	1,43	1,3	0,13
C4+ in test[1]	0,59	1,48	1,33	0,15
C5+ in test[1]	0,20	1,41	1,27	0,14
C6+ in test[1]	0,07	1,39	1,24	0,15
C7+ in test[1]				

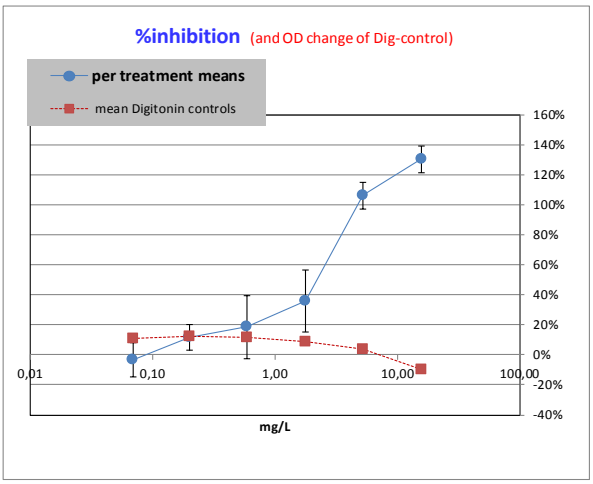
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,35	1,23	0,12
$C0_b$+	control1	1,42	1,24	0,18
C1+ in test[1]	15,91	1,43	1,57	-0,14
C2+ in test[1]	5,3	1,45	1,36	0,09
C3+ in test[1]	1,77	1,43	1,29	0,14
C4+ in test[1]	0,59	1,43	1,24	0,19
C5+ in test[1]	0,20	1,46	1,25	0,21
C6+ in test[1]	0,07	1,44	1,26	0,18
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,28	1,17	0,11
$C0_b$+	control1	1,37	1,21	0,16
C1+ in test[1]	15,91	1,38	1,55	-0,17
C2+ in test[1]	5,3	1,39	1,35	0,04
C3+ in test[1]	1,77	1,42	1,32	0,1
C4+ in test[1]	0,59	1,39	1,24	0,15
C5+ in test[1]	0,20	1,32	1,14	0,18
C6+ in test[1]	0,07	1,36	1,24	0,12
C7+ in test[1]				

[1]) including Digitonin



3.5-dichlorophenol: Lab_5_1 (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,353	0,768	0,585	0,033	0,001	6	**0,442**	**0,042**	**0%**	9%	✓ 33%
	mean C1	15,91	1,420	1,690	-0,270	0,020	0,0004	3	**-0,133**	**0,040**	**130%**	9%	-9%
	mean C2	5,30	1,427	1,397	0,030	0,026	0,0007	3	**-0,027**	**0,039**	**106%**	9%	-2%
	mean C3	1,77	1,420	1,013	0,407	0,087	0,0076	3	**0,283**	**0,090**	**36%**	20%	20%
	mean C4	0,59	1,397	0,873	0,523	0,091	0,0082	3	**0,360**	**0,094**	**18%**	21%	26%
	mean C5	0,20	1,400	0,833	0,567	0,015	0,0002	3	**0,390**	**0,038**	**12%**	9%	28%
	mean C6	0,070	1,427	0,820	0,607	0,040	0,0016	3	**0,457**	**0,050**	-3%	11%	32%
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_h$+	0[1]	1,373	1,230	0,143	0,0258	0,0007	6					✓ 10%
	mean C1+[1]	15,91	1,423	1,560	-0,137	0,035	0,0012333	3					✗ -10%
	mean C2+[1]	5,30	1,427	1,370	0,057	0,029	0,0008333	3					✓ 4%
	mean C3+[1]	1,77	1,427	1,303	0,123	0,021	0,0004333	3					✓ 9%
	mean C4+[1]	0,59	1,433	1,270	0,163	0,023	0,0005333	3					✓ 11%
	mean C5+[1]	0,20	1,397	1,220	0,177	0,035	0,0012333	3					✓ 13%
	mean C6+[1]	0,07	1,397	1,247	0,150	0,030	0,0009	3					✓ 11%
	mean C7+[1]	#NV											

[1]) including Digitonin

3.5-dichlorophenol: laboratory 5. test run 2 (Lab_5_2)

AS sampling date: 24-Jul-12
dry weight: 4,9 g/L

test date: 25-Jul-12
test substance: 3,5-Dichlorophenol

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

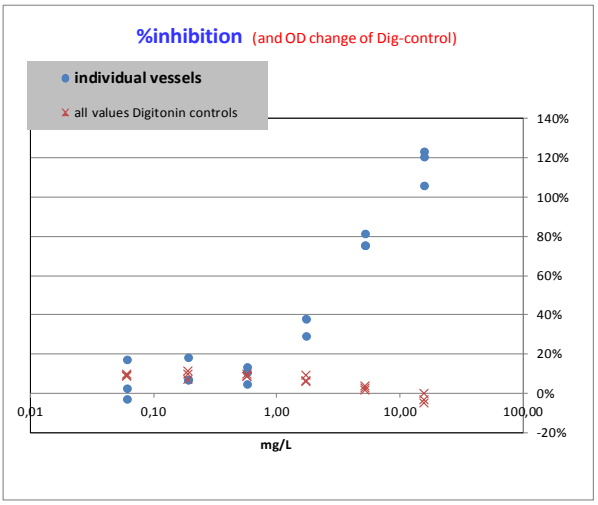
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,43	0,99	0,44
$C0_b$	control	1,42	0,92	0,5
C1 in test	15,52	1,37	1,49	-0,12
C2 in test	5,17	1,41	1,31	0,1
C3 in test	1,72	1,42	1,11	0,31
C4 in test	0,57	1,42	1,00	0,42
C5 in test	0,19	1,39	0,94	0,45
C6 in test	0,060	1,40	0,94	0,46
C7 in test				

Replicate 2

$C0_a$	control	1,38	0,94	0,44
$C0_b$	control	1,39	0,91	0,48
C1 in test	15,52	1,36	1,47	-0,11
C2 in test	5,17	1,42	1,3	0,12
C3 in test	1,72	1,38	1,07	0,31
C4 in test	0,57	1,38	0,93	0,45
C5 in test	0,19	1,38	0,93	0,45
C6 in test		1,36	0,95	0,41
C7 in test				

Replicate 3

$C0_a$	control	1,38	0,90	0,48
$C0_b$	control	1,36	0,90	0,46
C1 in test	15,52	1,41	1,47	-0,06
C2 in test	5,17	1,40	1,28	0,12
C3 in test	1,72	1,39	1,05	0,34
C4 in test	0,57	1,38	0,95	0,43
C5 in test	0,19	1,37	0,96	0,41
C6 in test	0,06	1,38	0,90	0,48
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,42	1,30	0,12
$C0_b$+	control1	1,42	1,29	0,13
C1+ in test[1]	15,52	1,43	1,43	0
C2+ in test[1]	5,17	1,44	1,4	0,04
C3+ in test[1]	1,72	1,4	1,27	0,13
C4+ in test[1]	0,57	1,42	1,28	0,14
C5+ in test[1]	0,19	1,44	1,28	0,16
C6+ in test[1]	0,06	1,42	1,28	0,14
C7+ in test[1]				

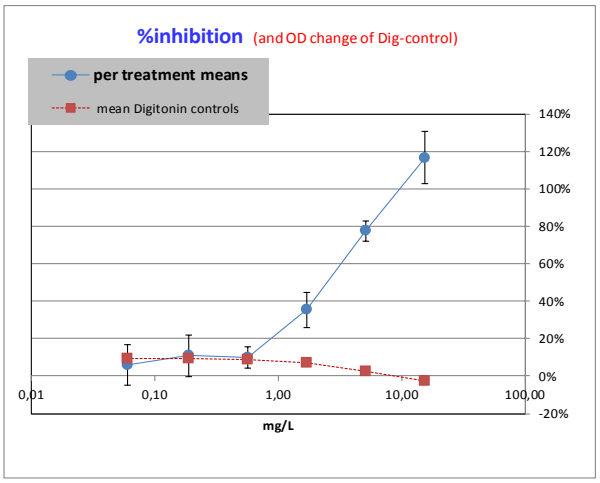
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,43	1,3	0,13
$C0_b$+	control1	1,41	1,31	0,1
C1+ in test[1]	15,52	1,43	1,5	-0,07
C2+ in test[1]	5,17	1,42	1,37	0,05
C3+ in test[1]	1,72	1,38	1,3	0,08
C4+ in test[1]	0,57	1,41	1,29	0,12
C5+ in test[1]	0,19	1,39	1,29	0,1
C6+ in test[1]	0,06	1,40	1,28	0,12
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,45	1,32	0,13
$C0_b$+	control1	1,43	1,28	0,15
C1+ in test[1]	15,52	1,44	1,49	-0,05
C2+ in test[1]	5,17	1,39	1,37	0,02
C3+ in test[1]	1,72	1,40	1,31	0,09
C4+ in test[1]	0,57	1,39	1,27	0,12
C5+ in test[1]	0,19	1,42	1,28	0,14
C6+ in test[1]	0,06	1,42	1,29	0,13
C7+ in test[1]				

[1]) including Digitonin



3.5-dichlorophenol: Lab_5_2 (continued)

	data analysis / evaluation											
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,393	0,927	0,467	0,024	0,001	6	**0,340**	**0,029**	**0%**	9%	✗ 24%
	mean C1	15,52	1,380	1,477	-0,097	0,032	0,0010	3	**-0,057**	**0,048**	**117%**	14%	-4%
	mean C2	5,17	1,410	1,297	0,113	0,012	0,0001	3	**0,077**	**0,019**	**77%**	6%	5%
	mean C3	1,72	1,397	1,077	0,320	0,017	0,0003	3	**0,220**	**0,032**	**35%**	9%	16%
	mean C4	0,57	1,393	0,960	0,433	0,015	0,0002	3	**0,307**	**0,019**	**10%**	6%	22%
	mean C5	0,19	1,380	0,943	0,437	0,023	0,0005	3	**0,303**	**0,038**	**11%**	11%	22%
	mean C6	0,060	1,380	0,930	0,450	0,036	0,0013	3	**0,320**	**0,037**	**6%**	11%	23%
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,427	1,300	0,127	0,0163	0,0003	6					✓ 9%
	mean C1+[1]	15,52	1,433	1,473	-0,040	0,036	0,0013	3					✓ -3%
	mean C2+[1]	5,17	1,417	1,380	0,037	0,015	0,0002333	3					✓ 3%
	mean C3+[1]	1,72	1,393	1,293	0,100	0,026	0,0007	3					✓ 7%
	mean C4+[1]	0,57	1,407	1,280	0,127	0,012	0,0001333	3					✓ 9%
	mean C5+[1]	0,19	1,417	1,283	0,133	0,031	0,0009333	3					✓ 9%
	mean C6+[1]	0,06	1,413	1,283	0,130	0,010	0,0001	3					✓ 9%
	mean C7+[1]	#NV											

[1]) including Digitonin

3.5-dichlorophenol: laboratory 5. test run 3 (Lab_5_3)

AS sampling date 07-Aug-12
dry weight 4,4 g/L

test date 08-Aug-12
test substance 3,5-dichlorophenol

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

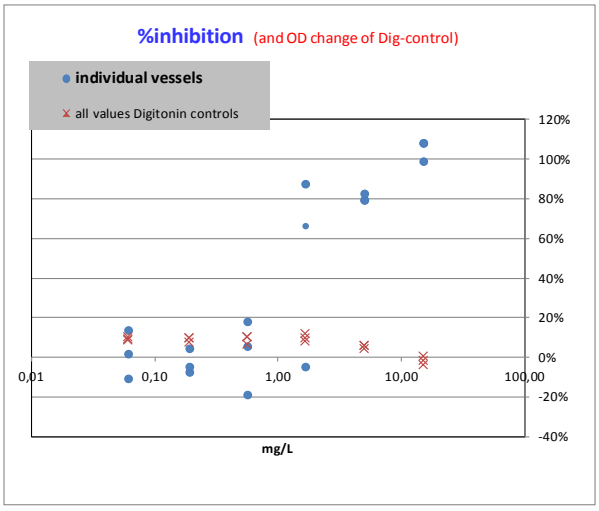
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,43	0,94	0,49
$C0_b$	control	1,46	0,91	0,55
C1 in test	15,05	1,48	1,53	-0,05
C2 in test	5,02	1,52	1,37	0,15
C3 in test	1,67	1,46	1,20	0,26
C4 in test	0,56	1,44	1,04	0,4
C5 in test	0,19	1,47	1,03	0,44
C6 in test	0,060	1,43	1,01	0,42
C7 in test				

Replicate 2

$C0_a$	control	1,44	0,95	0,49
$C0_b$	control	1,44	0,98	0,46
C1 in test	15,05	1,52	1,57	-0,05
C2 in test	5,02	1,48	1,33	0,15
C3 in test	1,67	1,37	1,18	0,19
C4 in test	0,56	1,41	0,97	0,44
C5 in test	0,19	1,45	0,97	0,48
C6 in test	0,06	1,41	0,95	0,46
C7 in test				

Replicate 3

$C0_a$	control	1,43	0,99	0,44
$C0_b$	control	1,43	0,98	0,45
C1 in test	15,05	1,52	1,54	-0,02
C2 in test	5,02	1,45	1,31	0,14
C3 in test	1,67	1,47	0,98	0,49
C4 in test	0,56	1,47	0,95	0,52
C5 in test	0,19	1,48	1,01	0,47
C6 in test	0,06	1,43	0,93	0,5
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,47	1,32	0,15
$C0_b$+	control1	1,48	1,31	0,17
C1+ in test[1]	15,05	1,58	1,64	-0,06
C2+ in test[1]	5,02	1,51	1,44	0,07
C3+ in test[1]	1,67	1,47	1,32	0,15
C4+ in test[1]	0,56	1,45	1,3	0,15
C5+ in test[1]	0,19	1,45	1,31	0,14
C6+ in test[1]	0,06	1,48	1,35	0,13
C7+ in test[1]				

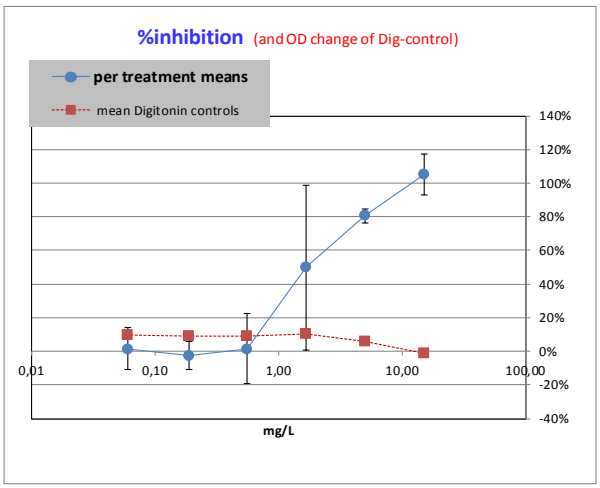
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,46	1,31	0,15
$C0_b$+	control1	1,46	1,29	0,17
C1+ in test[1]	15,05	1,58	1,6	-0,02
C2+ in test[1]	5,02	1,5	1,41	0,09
C3+ in test[1]	1,67	1,49	1,37	0,12
C4+ in test[1]	0,56	1,47	1,32	0,15
C5+ in test[1]	0,19	1,43	1,32	0,11
C6+ in test[1]	0,06	1,47	1,33	0,14
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,47	1,32	0,15
$C0_b$+	control1	1,48	1,34	0,14
C1+ in test[1]	15,05	1,59	1,58	0,01
C2+ in test[1]	5,02	1,50	1,41	0,09
C3+ in test[1]	1,67	1,48	1,30	0,18
C4+ in test[1]	0,56	1,47	1,37	0,1
C5+ in test[1]	0,19	1,46	1,32	0,14
C6+ in test[1]	0,06	1,47	1,32	0,15
C7+ in test[1]				

[1]) including Digitonin



3.5-dichlorophenol: Lab_5_3 (continued)

data analysis / evaluation

	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,438	0,958	0,480	0,040	0,002	6	0,325	0,042	0%	13%	✗ 23%
	mean C1	15,05	1,507	1,547	-0,040	0,017	0,0003	3	-0,017	0,039	105%	12%	-1%
	mean C2	5,02	1,483	1,337	0,147	0,006	0,0000	3	0,063	0,013	81%	4%	4%
	mean C3	1,67	1,433	1,120	0,313	0,157	0,0246	3	0,163	0,160	50%	49%	11%
	mean C4	0,56	1,440	0,987	0,453	0,061	0,0037	3	0,320	0,068	2%	21%	22%
	mean C5	0,19	1,467	1,003	0,463	0,021	0,0004	3	0,333	0,027	-3%	8%	23%
	mean C6	0,060	1,423	0,963	0,460	0,040	0,0016	3	0,320	0,041	2%	13%	22%
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,470	1,315	0,155	0,0122	0,0002	6					✓ 11%
	mean C1+[1]	15,05	1,583	1,607	-0,023	0,035	0,0012333	3					✓ -1%
	mean C2+[1]	5,02	1,503	1,420	0,083	0,012	0,0001333	3					✓ 6%
	mean C3+[1]	1,67	1,480	1,330	0,150	0,030	0,0009	3					✓ 10%
	mean C4+[1]	0,56	1,463	1,330	0,133	0,029	0,0008333	3					✓ 9%
	mean C5+[1]	0,19	1,447	1,317	0,130	0,017	0,0003	3					✓ 9%
	mean C6+[1]	0,06	1,473	1,333	0,140	0,010	0,0001	3					✓ 10%
	mean C7+[1]	#NV											

[1]) including Digitonin

Dimethyl sulfoxide: laboratory I. test run I (Lab_I_I)

AS sampling date: 28-Feb-13
dry weight: 3,6 g/L

test date: 28-Feb-13
test substance: DMSO

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

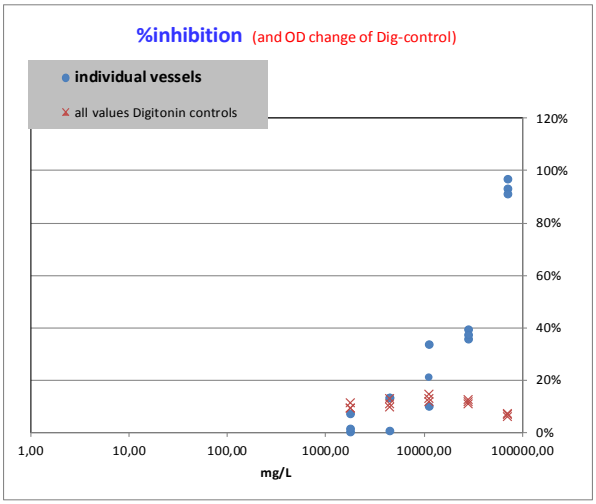
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,30	0,13	1,17
$C0_b$	control	1,39	0,16	1,23
C1 in test	70222,22	1,24	1,12	0,12
C2 in test	28088,89	1,28	0,45	0,83
C3 in test	11235,56	1,29	0,29	1
C4 in test	4494,22	1,25	0,20	1,05
C5 in test	1797,69	1,26	0,09	1,17
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,27	0,08	1,19
$C0_b$	control	1,24	0,07	1,17
C1 in test	70222,22	1,32	1,16	0,16
C2 in test	28088,89	1,27	0,48	0,79
C3 in test	11235,56	1,25	0,38	0,87
C4 in test	4494,22	1,26	0,21	1,05
C5 in test	1797,69	1,26	0,1	1,16
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,25	0,08	1,17
$C0_b$	control	1,36	0,14	1,22
C1 in test	70222,22	1,34	1,16	0,18
C2 in test	28088,89	1,31	0,50	0,81
C3 in test	11235,56	1,29	0,18	1,11
C4 in test	4494,22	1,31	0,13	1,18
C5 in test	1797,69	1,32	0,22	1,1
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,53	1,33	0,2
$C0_b$+	control1	1,50	1,35	0,15
C1+ in test[1]	70222,22	1,38	1,29	0,09
C2+ in test[1]	28088,89	1,46	1,3	0,16
C3+ in test[1]	11235,56	1,49	1,32	0,17
C4+ in test[1]	4494,22	1,46	1,32	0,14
C5+ in test[1]	1797,69	1,51	1,34	0,17
C6+ in test[1]				
C7+ in test[1]				

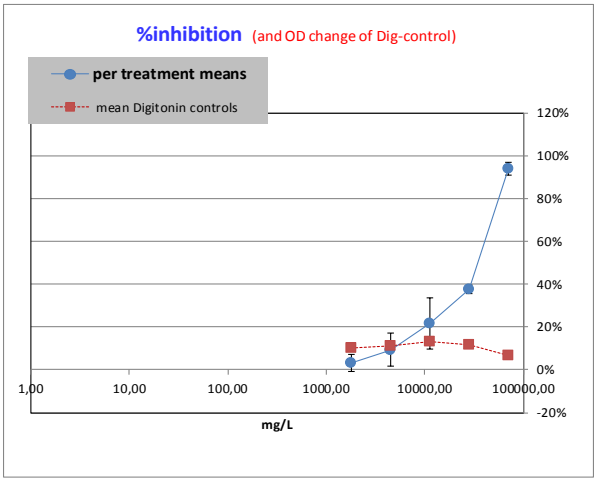
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,51	1,31	0,2
$C0_b$+	control1	1,5	1,35	0,15
C1+ in test[1]	70222,22	1,29	1,21	0,08
C2+ in test[1]	28088,89	1,44	1,26	0,18
C3+ in test[1]	11235,56	1,46	1,27	0,19
C4+ in test[1]	4494,22	1,45	1,26	0,19
C5+ in test[1]	1797,69	1,44	1,3	0,14
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,46	1,34	0,12
$C0_b$+	control1	1,54	1,36	0,18
C1+ in test[1]	70222,22	1,37	1,27	0,1
C2+ in test[1]	28088,89	1,43	1,26	0,17
C3+ in test[1]	11235,56	1,43	1,22	0,21
C4+ in test[1]	4494,22	1,46	1,30	0,16
C5+ in test[1]	1797,69	1,48	1,34	0,14
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



Dimethyl sulfoxide: Lab_1_1 (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,302	0,110	1,192	0,027	0,001	6	**1,025**	**0,042**	**0%**	4%	✓ 79%
	mean C1	70222,22	1,300	1,147	0,153	0,031	0,0009	3	**0,063**	**0,032**	**94%**	3%	5%
	mean C2	28088,89	1,287	0,477	0,810	0,020	0,0004	3	**0,640**	**0,022**	**38%**	2%	50%
	mean C3	11235,56	1,277	0,283	0,993	0,120	0,0144	3	**0,803**	**0,122**	**22%**	12%	63%
	mean C4	4494,22	1,273	0,180	1,093	0,075	0,0056	3	**0,930**	**0,079**	**9%**	8%	73%
	mean C5	1797,69	1,280	0,137	1,143	0,038	0,0014	3	**0,993**	**0,042**	**3%**	4%	78%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,507	1,340	0,167	0,0320	0,0010	6					✓ 11%
	mean C1+[1]	70222,22	1,347	1,257	0,090	0,010	0,0001	3					✓ 7%
	mean C2+[1]	28088,89	1,443	1,273	0,170	0,010	0,0001	3					✓ 12%
	mean C3+[1]	11235,56	1,460	1,270	0,190	0,020	0,0004	3					✓ 13%
	mean C4+[1]	4494,22	1,457	1,293	0,163	0,025	0,0006333	3					✓ 11%
	mean C5+[1]	1797,69	1,477	1,327	0,150	0,017	0,0003	3					✓ 10%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

Dimethyl sulfoxide: laboratory 2. test run 1 (Lab_2_1)

AS sampling date 18-Jan-12
dry weight 2,09 g/L

test date 21-Jan-13
test substance Dimethyl sulfoxid

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

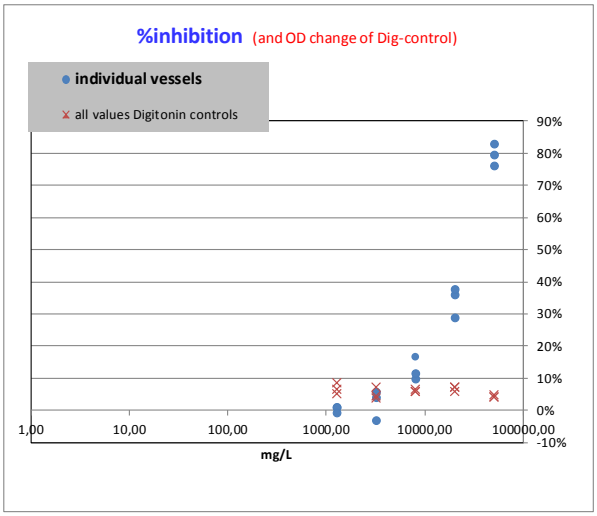
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,32	0,67	0,65
$C0_b$	control	1,42	0,73	0,69
C1 in test	50153,11	1,40	1,24	0,16
C2 in test	20061,24	1,39	0,88	0,51
C3 in test	8024,50	1,39	0,82	0,57
C4 in test	3209,80	1,41	0,78	0,63
C5 in test	1283,92	1,41	0,74	0,67
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,4	0,72	0,68
$C0_b$	control	1,42	0,78	0,64
C1 in test	50153,11	1,4	1,2	0,2
C2 in test	20061,24	1,39	0,92	0,47
C3 in test	8024,50	1,41	0,8	0,61
C4 in test	3209,80	1,43	0,76	0,67
C5 in test	1283,92	1,42	0,75	0,67
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,43	0,78	0,65
$C0_b$	control	1,42	0,72	0,7
C1 in test	50153,11	1,38	1,20	0,18
C2 in test	20061,24	1,39	0,93	0,46
C3 in test	8024,50	1,41	0,81	0,6
C4 in test	3209,80	1,41	0,79	0,62
C5 in test	1283,92	1,36	0,68	0,68
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,54	1,43	0,11
$C0_b$+	control1	1,52	1,43	0,09
C1+ in test[1]	50153,11	1,43	1,36	0,07
C2+ in test[1]	20061,24	1,47	1,36	0,11
C3+ in test[1]	8024,5	1,5	1,4	0,1
C4+ in test[1]	3209,80	1,54	1,43	0,11
C5+ in test[1]	1283,92	1,54	1,41	0,13
C6+ in test[1]				
C7+ in test[1]				

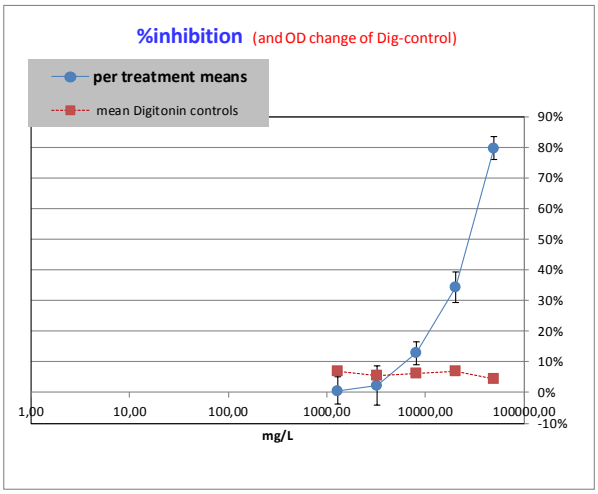
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,48	1,38	0,1
$C0_b$+	control1	1,52	1,42	0,1
C1+ in test[1]	50153,11	1,45	1,39	0,06
C2+ in test[1]	20061,24	1,5	1,41	0,09
C3+ in test[1]	8024,5	1,5	1,41	0,09
C4+ in test[1]	3209,8	1,48	1,41	0,07
C5+ in test[1]	1283,92	1,53	1,45	0,08
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,52	1,43	0,09
$C0_b$+	control1	1,52	1,44	0,08
C1+ in test[1]	50153,11	1,43	1,37	0,06
C2+ in test[1]	20061,24	1,50	1,39	0,11
C3+ in test[1]	8024,5	1,50	1,41	0,09
C4+ in test[1]	3209,80	1,49	1,43	0,06
C5+ in test[1]	1283,92	1,51	1,41	0,1
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



Dimethyl sulfoxide: Lab_2_1 (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,402	0,733	0,668	0,025	0,001	6	**0,573**	**0,027**	**0%**	5%	✓ 41%
mean C1	50153,11	1,393	1,213	0,180	0,020	0,0004	3	**0,117**	**0,021**	**80%**	4%	8%
mean C2	20061,24	1,390	0,910	0,480	0,026	0,0007	3	**0,377**	**0,029**	**34%**	5%	27%
mean C3	8024,50	1,403	0,810	0,593	0,021	0,0004	3	**0,500**	**0,022**	**13%**	4%	36%
mean C4	3209,80	1,417	0,777	0,640	0,026	0,0007	3	**0,560**	**0,037**	**2%**	7%	40%
mean C5	1283,92	1,397	0,723	0,673	0,006	0,0000	3	**0,570**	**0,026**	**1%**	5%	41%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
mean C7	#NV										0%	

Replicate2 1-3

mean $C0_b$+	0[1]	1,517	1,422	0,095	0,0105	0,0001	6					✓ 6%
mean C1+[1]	50153,11	1,437	1,373	0,063	0,006	3,333E-05	3					✓ 4%
mean C2+[1]	20061,24	1,490	1,387	0,103	0,012	0,0001333	3					✓ 7%
mean C3+[1]	8024,50	1,500	1,407	0,093	0,006	3,333E-05	3					✓ 6%
mean C4+[1]	3209,80	1,503	1,423	0,080	0,026	0,0007	3					✓ 5%
mean C5+[1]	1283,92	1,527	1,423	0,103	0,025	0,0006333	3					✓ 7%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

Dimethyl sulfoxide: laboratory 3. test run 1 (Lab_3_1)

AS sampling date: 14-Feb-13
dry weight: 5,32 g/L

test date: 14-Feb-13
test substance: Dimethyl sulfoxide

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

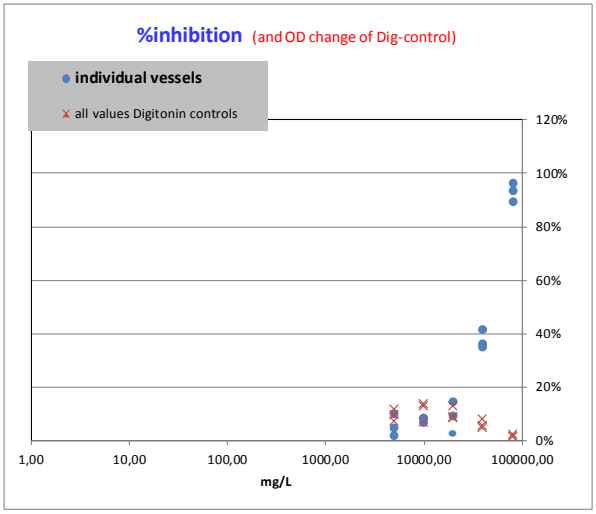
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,18	0,32	0,86
$C0_b$	control	1,70	0,81	0,89
C1 in test	79203,01	1,39	1,32	0,07
C2 in test	39601,50	1,19	0,64	0,55
C3 in test	19800,75	1,19	0,33	0,86
C4 in test	9900,38	1,06	0,22	0,84
C5 in test	4950,19	0,98	0,14	0,84
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,09	0,24	0,85
$C0_b$	control	1,12	0,26	0,86
C1 in test	79203,01	1,15	1,05	0,1
C2 in test	39601,50	1,2	0,69	0,51
C3 in test	19800,75	1,12	0,31	0,81
C4 in test	9900,38	1,1	0,27	0,83
C5 in test	4950,19	1,04	0,24	0,8
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,15	0,24	0,91
$C0_b$	control	1,10	0,11	0,99
C1 in test	79203,01	1,17	1,12	0,05
C2 in test	39601,50	1,17	0,61	0,56
C3 in test	19800,75	1,38	0,61	0,77
C4 in test	9900,38	1,25	0,42	0,83
C5 in test	4950,19	1,11	0,25	0,86
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,39	1,26	0,13
$C0_b$+	control1	1,32	1,17	0,15
C1+ in test[1]	79203,01	1,23	1,21	0,02
C2+ in test[1]	39601,5	1,25	1,15	0,1
C3+ in test[1]	19800,75	1,39	1,27	0,12
C4+ in test[1]	9900,38	1,37	1,2	0,17
C5+ in test[1]	4950,19	1,28	1,15	0,13
C6+ in test[1]				
C7+ in test[1]				

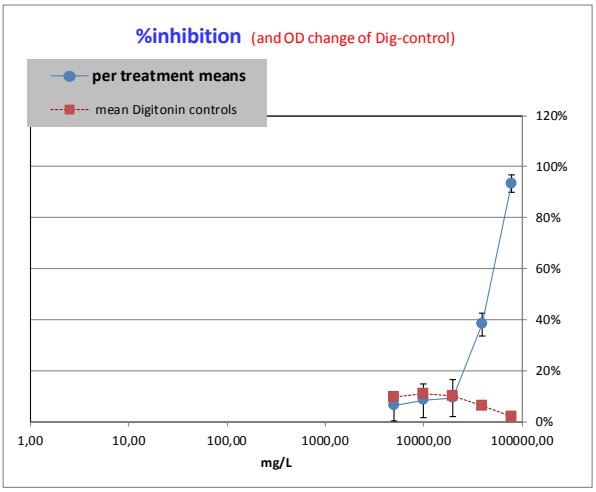
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,39	1,28	0,11
$C0_b$+	control1	1,32	1,17	0,15
C1+ in test[1]	79203,01	1,23	1,21	0,02
C2+ in test[1]	39601,5	1,26	1,2	0,06
C3+ in test[1]	19800,75	1,33	1,16	0,17
C4+ in test[1]	9900,38	1,31	1,13	0,18
C5+ in test[1]	4950,19	1,46	1,3	0,16
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,37	1,23	0,14
$C0_b$+	control1	1,30	1,11	0,19
C1+ in test[1]	79203,01	1,32	1,29	0,03
C2+ in test[1]	39601,5	1,25	1,18	0,07
C3+ in test[1]	19800,75	1,25	1,14	0,11
C4+ in test[1]	9900,38	1,29	1,20	0,09
C5+ in test[1]	4950,19	1,32	1,22	0,1
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



Dimethyl sulfoxide: Lab_3_1 (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,223	0,330	0,893	0,052	0,003	6	**0,748**	**0,059**	**0%**	8%	✓ 61%
mean C1	79203,01	1,237	1,163	0,073	0,025	0,0006	3	**0,050**	**0,026**	**93%**	3%	4%
mean C2	39601,50	1,187	0,647	0,540	0,026	0,0007	3	**0,463**	**0,034**	**38%**	4%	39%
mean C3	19800,75	1,230	0,417	0,813	0,045	0,0020	3	**0,680**	**0,055**	**9%**	7%	55%
mean C4	9900,38	1,137	0,303	0,833	0,006	0,0000	3	**0,687**	**0,050**	**8%**	7%	60%
mean C5	4950,19	1,043	0,210	0,833	0,031	0,0009	3	**0,703**	**0,043**	**6%**	6%	67%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
mean C7	#NV										**0%**	

Replicate2 1-3

mean $C0_b$+	0[1]	1,348	1,203	0,145	0,0266	0,0007	6					✓ 11%
mean C1+[1]	79203,01	1,260	1,237	0,023	0,006	3,333E-05	3					✓ 2%
mean C2+[1]	39601,50	1,253	1,177	0,077	0,021	0,0004333	3					✓ 6%
mean C3+[1]	19800,75	1,323	1,190	0,133	0,032	0,0010333	3					✓ 10%
mean C4+[1]	9900,38	1,323	1,177	0,147	0,049	0,0024333	3					✓ 11%
mean C5+[1]	4950,19	1,353	1,223	0,130	0,030	0,0009	3					✓ 10%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+ Digitonin)

[1]) including Digitonin

Dimethyl sulfoxide: laboratory 4. test run 1 (Lab_4_1)

AS sampling date: 31-Aug-12
dry weight: 3,5 g/L

test date: 02-Sep-12
test substance: Dimethyl sulfoxid

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

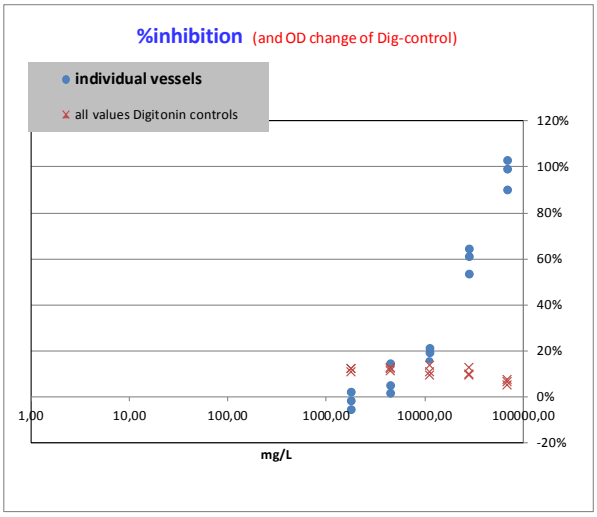
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,41	0,72	0,69
$C0_b$	control	1,39	0,69	0,7
C1 in test	69428,57	1,40	1,31	0,09
C2 in test	27771,43	1,39	1,03	0,36
C3 in test	11108,57	1,38	0,77	0,61
C4 in test	4443,43	1,41	0,71	0,7
C5 in test	1777,37	1,37	0,66	0,71
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,39	0,7	0,69
$C0_b$	control	1,42	0,73	0,69
C1 in test	69428,57	1,41	1,34	0,07
C2 in test	27771,43	1,39	1,05	0,34
C3 in test	11108,57	1,43	0,84	0,59
C4 in test	4443,43	1,39	0,76	0,63
C5 in test	1777,37	1,4	0,71	0,69
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,38	0,68	0,7
$C0_b$	control	1,39	0,70	0,69
C1 in test	69428,57	1,37	1,23	0,14
C2 in test	27771,43	1,40	1,00	0,4
C3 in test	11108,57	1,39	0,81	0,58
C4 in test	4443,43	1,37	0,69	0,68
C5 in test	1777,37	1,38	0,65	0,73
C6 in test				
C7 in test				

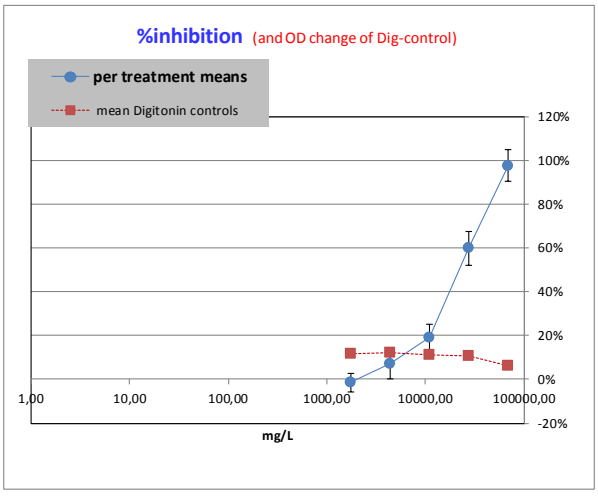


Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,40	1,23	0,17
$C0_b$+	control1	1,38	1,22	0,16
C1+ in test[1]	69428,57	1,38	1,29	0,09
C2+ in test[1]	27771,43	1,39	1,25	0,14
C3+ in test[1]	11108,57	1,41	1,22	0,19
C4+ in test[1]	4443,43	1,4	1,24	0,16
C5+ in test[1]	1777,37	1,39	1,22	0,17
C6+ in test[1]				
C7+ in test[1]				

Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,37	1,19	0,18
$C0_b$+	control1	1,41	1,24	0,17
C1+ in test[1]	69428,57	1,37	1,27	0,1
C2+ in test[1]	27771,43	1,4	1,22	0,18
C3+ in test[1]	11108,57	1,39	1,24	0,15
C4+ in test[1]	4443,43	1,37	1,19	0,18
C5+ in test[1]	1777,37	1,36	1,19	0,17
C6+ in test[1]				
C7+ in test[1]				



Replicate 3 (+Digitonin)

$C0_a$+	control1	1,42	1,30	0,12
$C0_b$+	control1	1,39	1,26	0,13
C1+ in test[1]	69428,57	1,38	1,31	0,07
C2+ in test[1]	27771,43	1,39	1,26	0,13
C3+ in test[1]	11108,57	1,40	1,27	0,13
C4+ in test[1]	4443,43	1,41	1,24	0,17
C5+ in test[1]	1777,37	1,38	1,23	0,15
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin

Dimethyl sulfoxide: Lab_4_1 (continued)

	data analysis / evaluation											
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,397	0,703	0,693	0,005	0,000	6	0,538	0,025	0%	5%	✓ 39%
	mean C1	69428,57	1,393	1,293	0,100	0,036	0,0013	3	0,013	0,039	98%	7%	1%
	mean C2	27771,43	1,393	1,027	0,367	0,031	0,0009	3	0,217	0,040	60%	8%	16%
	mean C3	11108,57	1,400	0,807	0,593	0,015	0,0002	3	0,437	0,034	19%	6%	31%
	mean C4	4443,43	1,390	0,720	0,670	0,036	0,0013	3	0,500	0,037	7%	7%	36%
	mean C5	1777,37	1,383	0,673	0,710	0,020	0,0004	3	0,547	0,023	-2%	4%	40%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,395	1,240	0,155	0,0243	0,0006	6					✓ 11%
	mean C1+[1]	69428,57	1,377	1,290	0,087	0,015	0,0002333	3					✓ 6%
	mean C2+[1]	27771,43	1,393	1,243	0,150	0,026	0,0007	3					✓ 11%
	mean C3+[1]	11108,57	1,400	1,243	0,157	0,031	0,0009333	3					✓ 11%
	mean C4+[1]	4443,43	1,393	1,223	0,170	0,010	0,0001	3					✓ 12%
	mean C5+[1]	1777,37	1,377	1,213	0,163	0,012	0,0001333	3					✓ 12%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

Dimethyl sulfoxide: laboratory 5. test run 1 (Lab_5_1)

AS sampling date	17-Jul-12
dry weight	4,4 g/L
test date	19-Jul-12
test substance	Dimethyl sulfoxide

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2	.	C2+
	C3	.	C3+
	C4	.	C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

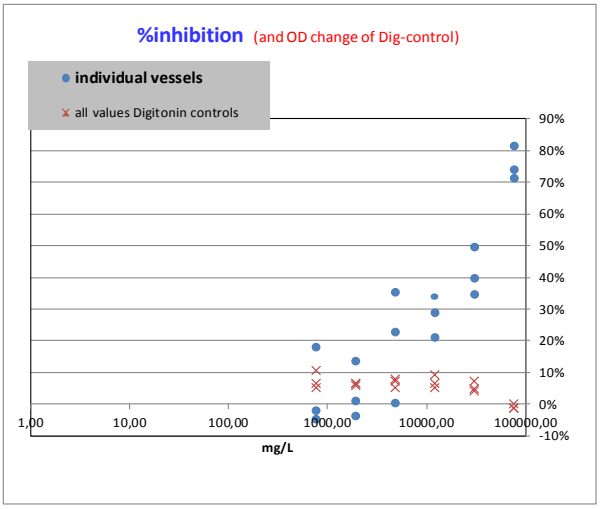
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,50	1,00	0,5
$C0_b$	control	1,54	1,01	0,53
C1 in test	75272,73	1,40	1,31	0,09
C2 in test	30109,09	1,45	1,17	0,28
C3 in test	12043,64	1,49	1,12	0,37
C4 in test	4817,45	1,50	1,09	0,41
C5 in test	1926,98	1,50	1,06	0,44
C6 in test	770,790	1,44	1,00	0,44
C7 in test				

Replicate 2

$C0_a$	control	1,5	0,97	0,53
$C0_b$	control	1,52	1,01	0,51
C1 in test	75272,73	1,44	1,38	0,06
C2 in test	30109,09	1,49	1,15	0,34
C3 in test	12043,64	1,52	1,1	0,42
C4 in test	4817,45	1,47	1,11	0,36
C5 in test	1926,98	1,55	1,06	0,49
C6 in test	770,79	1,49	0,97	0,52
C7 in test				

Replicate 3

$C0_a$	control	1,52	1,04	0,48
$C0_b$	control	1,53	1,01	0,52
C1 in test	75272,73	1,45	1,35	0,1
C2 in test	30109,09	1,46	1,14	0,32
C3 in test	12043,64	1,49	1,10	0,39
C4 in test	4817,45	1,54	1,04	0,5
C5 in test	1926,98	1,52	1,01	0,51
C6 in test	770,79	1,51	0,98	0,53
C7 in test				

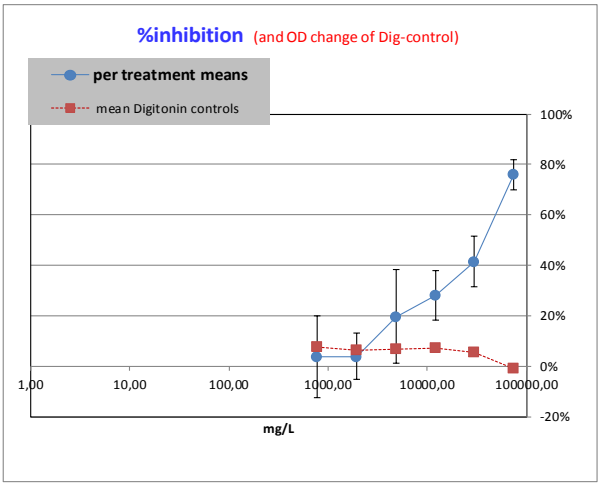


Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,52	1,38	0,14
$C0_b$+	control1	1,51	1,43	0,08
C1+ in test[1]	75272,73	1,44	1,46	-0,02
C2+ in test[1]	30109,09	1,48	1,41	0,07
C3+ in test[1]	12043,64	1,49	1,41	0,08
C4+ in test[1]	4817,45	1,52	1,4	0,12
C5+ in test[1]	1926,98	1,51	1,42	0,09
C6+ in test[1]	770,79	1,50	1,42	0,08
C7+ in test[1]				

Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,52	1,43	0,09
$C0_b$+	control1	1,52	1,37	0,15
C1+ in test[1]	75272,73	1,43	1,45	-0,02
C2+ in test[1]	30109,09	1,47	1,41	0,06
C3+ in test[1]	12043,64	1,5	1,4	0,1
C4+ in test[1]	4817,45	1,52	1,41	0,11
C5+ in test[1]	1926,98	1,51	1,41	0,1
C6+ in test[1]	770,79	1,54	1,38	0,16
C7+ in test[1]				



Replicate 3 (+Digitonin)

$C0_a$+	control1	1,54	1,42	0,12
$C0_b$+	control1	1,50	1,40	0,1
C1+ in test[1]	75272,73	1,44	1,44	0
C2+ in test[1]	30109,09	1,51	1,40	0,11
C3+ in test[1]	12043,64	1,52	1,38	0,14
C4+ in test[1]	4817,45	1,49	1,41	0,08
C5+ in test[1]	1926,98	1,49	1,39	0,1
C6+ in test[1]	770,79	1,50	1,40	0,1
C7+ in test[1]				

[1]) including Digitonin

Dimethyl sulfoxide: Lab_5_1 (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
mean C0	0	1,518	1,007	0,512	0,019	0,000	6	**0,398**	**0,034**	**0%**	9%	✓ **26%**
mean C1	75272,73	1,430	1,347	0,083	0,021	0,0004	3	**0,097**	**0,024**	**76%**	6%	7%
mean C2	30109,09	1,467	1,153	0,313	0,031	0,0009	3	**0,233**	**0,040**	**41%**	10%	16%
mean C3	12043,64	1,500	1,107	0,393	0,025	0,0006	3	**0,287**	**0,040**	**28%**	10%	19%
mean C4	4817,45	1,503	1,080	0,423	0,071	0,0050	3	**0,320**	**0,074**	**20%**	19%	21%
mean C5	1926,98	1,523	1,043	0,480	0,036	0,0013	3	**0,383**	**0,037**	**4%**	9%	25%
mean C6	770,790	1,480	0,983	0,497	0,049	0,0024	3	**0,383**	**0,065**	**4%**	16%	26%
mean C7	#NV										**0%**	
Replicate2 1-3												
mean $C0_b$+	0[1]	1,518	1,405	0,113	0,0280	0,0008	6					✓ 7%
mean C1+[1]	75272,73	1,437	1,450	-0,013	0,012	0,0001333	3					✓ -1%
mean C2+[1]	30109,09	1,487	1,407	0,080	0,026	0,0007	3					✓ 5%
mean C3+[1]	12043,64	1,503	1,397	0,107	0,031	0,0009333	3					✓ 7%
mean C4+[1]	4817,45	1,510	1,407	0,103	0,021	0,0004333	3					✓ 7%
mean C5+[1]	1926,98	1,503	1,407	0,097	0,006	3,333E-05	3					✓ 6%
mean C6+[1]	770,79	1,513	1,400	0,113	0,042	0,001733333	3					✓ 7%
mean C7+[1]	#NV											
Replicate 1-3 (+Digitonin)												

[1]) including Digitonin

Dimethyl sulfoxide: laboratory 5. test run 2 (Lab_5_2)

AS sampling date: 07-Aug-12
dry weight: 4,4 g/L

test date: 09-Aug-12
test substance: dimethyl sulfoxide

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

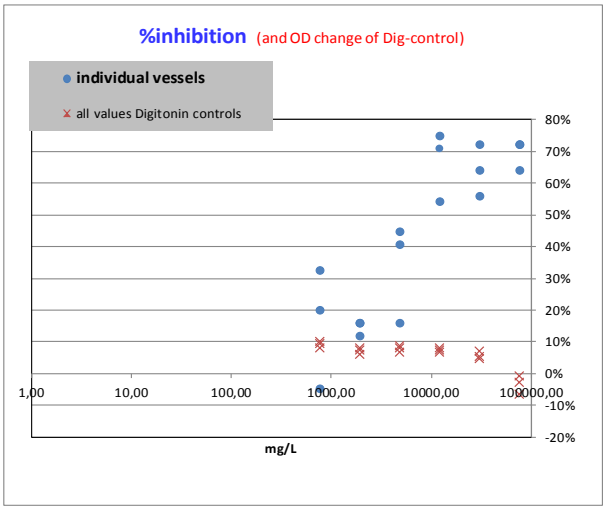
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,45	1,06	0,39
$C0_b$	control	1,45	1,09	0,36
C1 in test	75272,73	1,43	1,41	0,02
C2 in test	30109,09	1,41	1,26	0,15
C3 in test	12043,64	1,39	1,21	0,18
C4 in test	4817,45	1,35	1,10	0,25
C5 in test	1926,98	1,47	1,15	0,32
C6 in test	770,790	1,40	1,10	0,3
C7 in test				

Replicate 2

$C0_a$	control	1,46	1,09	0,37
$C0_b$	control	1,42	1,06	0,36
C1 in test	75272,73	1,48	1,44	0,04
C2 in test	30109,09	1,42	1,25	0,17
C3 in test	12043,64	1,41	1,24	0,17
C4 in test	4817,45	1,47	1,15	0,32
C5 in test	1926,98	1,44	1,13	0,31
C6 in test	770,79	1,46	1,13	0,33
C7 in test				

Replicate 3

$C0_a$	control	1,46	1,10	0,36
$C0_b$	control	1,47	1,10	0,37
C1 in test	75272,73	1,44	1,42	0,02
C2 in test	30109,09	1,44	1,25	0,19
C3 in test	12043,64	1,45	1,23	0,22
C4 in test	4817,45	1,42	1,16	0,26
C5 in test	1926,98	1,39	1,08	0,31
C6 in test	770,79	1,46	1,07	0,39
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,52	1,38	0,14
$C0_b$+	control1	1,55	1,42	0,13
C1+ in test[1]	75272,73	1,37	1,41	-0,04
C2+ in test[1]	30109,09	1,44	1,34	0,1
C3+ in test[1]	12043,64	1,46	1,36	0,1
C4+ in test[1]	4817,45	1,45	1,35	0,1
C5+ in test[1]	1926,98	1,47	1,35	0,12
C6+ in test[1]	770,79	1,47	1,35	0,12
C7+ in test[1]				

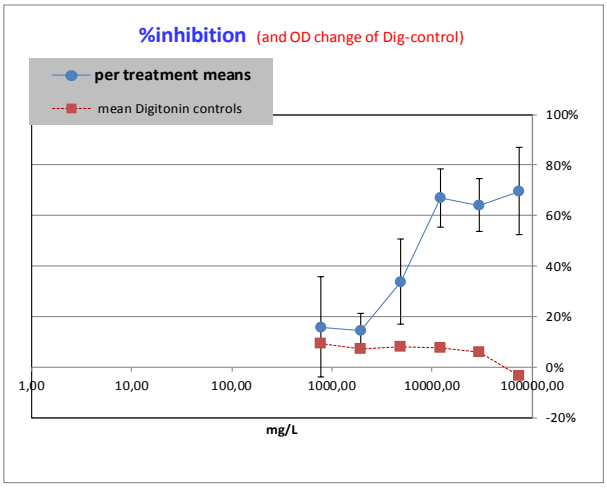
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,52	1,4	0,12
$C0_b$+	control1	1,5	1,39	0,11
C1+ in test[1]	75272,73	1,42	1,43	-0,01
C2+ in test[1]	30109,09	1,43	1,36	0,07
C3+ in test[1]	12043,64	1,46	1,35	0,11
C4+ in test[1]	4817,45	1,47	1,35	0,12
C5+ in test[1]	1926,98	1,53	1,42	0,11
C6+ in test[1]	770,79	1,50	1,35	0,15
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,54	1,38	0,16
$C0_b$+	control1	1,50	1,40	0,1
C1+ in test[1]	75272,73	1,35	1,44	-0,09
C2+ in test[1]	30109,09	1,44	1,36	0,08
C3+ in test[1]	12043,64	1,45	1,33	0,12
C4+ in test[1]	4817,45	1,50	1,37	0,13
C5+ in test[1]	1926,98	1,45	1,36	0,09
C6+ in test[1]	770,79	1,52	1,38	0,14
C7+ in test[1]				

[1]) including Digitonin



3 Dimethyl sulfoxide: Lab_5_2 (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,452	1,083	0,368	0,012	0,000	6	**0,242**	**0,025**	**0%**	10%	✗ 17%
	mean C1	75272,73	1,450	1,423	0,027	0,012	0,0001	3	**0,073**	**0,042**	**70%**	17%	5%
	mean C2	30109,09	1,423	1,253	0,170	0,020	0,0004	3	**0,087**	**0,025**	**64%**	10%	6%
	mean C3	12043,64	1,417	1,227	0,190	0,026	0,0007	3	**0,080**	**0,028**	**67%**	12%	6%
	mean C4	4817,45	1,413	1,137	0,277	0,038	0,0014	3	**0,160**	**0,041**	**34%**	17%	11%
	mean C5	1926,98	1,433	1,120	0,313	0,006	0,0000	3	**0,207**	**0,016**	**14%**	7%	14%
	mean C6	770,790	1,440	1,100	0,340	0,046	0,0021	3	**0,203**	**0,048**	**16%**	20%	14%
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,522	1,395	0,127	0,0216	0,0005	6					✓ 8%
	mean C1+[1]	75272,73	1,380	1,427	-0,047	0,040	0,0016333	3					✓ -3%
	mean C2+[1]	30109,09	1,437	1,353	0,083	0,015	0,0002333	3					✓ 6%
	mean C3+[1]	12043,64	1,457	1,347	0,110	0,010	0,0001	3					✓ 8%
	mean C4+[1]	4817,45	1,473	1,357	0,117	0,015	0,0002333	3					✓ 8%
	mean C5+[1]	1926,98	1,483	1,377	0,107	0,015	0,0002333	3					✓ 7%
	mean C6+[1]	770,79	1,497	1,360	0,137	0,015	0,000233333	3					✓ 9%
	mean C7+[1]	#NV											

[1]) including Digitonin

hexachlorophene: laboratory I. test run I (Lab_I_I)

AS sampling date: 11-Apr-13
dry weight: 3,1 g/L

test date: 12-Apr-13
test substance: hexachlorophen

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

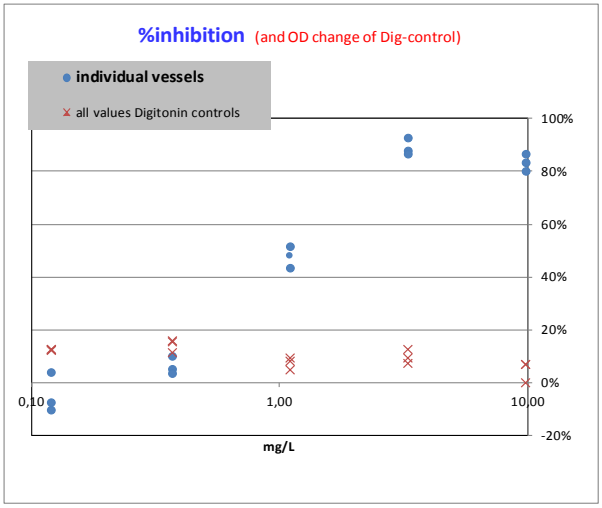
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,40	0,78	0,62
$C0_b$	control	1,38	0,52	0,86
C1 in test	9,86	1,50	1,35	0,15
C2 in test	3,29	1,48	1,26	0,22
C3 in test	1,10	1,40	0,97	0,43
C4 in test	0,37	1,38	0,61	0,77
C5 in test	0,12	1,39	0,61	0,78
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,37	0,46	0,91
$C0_b$	control	1,42	0,55	0,87
C1 in test	9,86	1,53	1,36	0,17
C2 in test	3,29	1,48	1,25	0,23
C3 in test	1,10	1,4	0,99	0,41
C4 in test	0,37	1,39	0,59	0,8
C5 in test	0,12	1,36	0,49	0,87
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,35	0,51	0,84
$C0_b$	control	1,37	0,57	0,8
C1 in test	9,86	1,54	1,35	0,19
C2 in test	3,29	1,47	1,28	0,19
C3 in test	1,10	1,49	1,03	0,46
C4 in test	0,37	1,40	0,59	0,81
C5 in test	0,12	1,39	0,54	0,85
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,49	1,25	0,24
$C0_b$+	control1	1,52	1,28	0,24
C1+ in test[1]	9,86	1,53	1,43	0,1
C2+ in test[1]	3,29	1,54	1,35	0,19
C3+ in test[1]	1,1	1,43	1,36	0,07
C4+ in test[1]	0,37	1,51	1,27	0,24
C5+ in test[1]	0,12	1,48	1,29	0,19
C6+ in test[1]				
C7+ in test[1]				

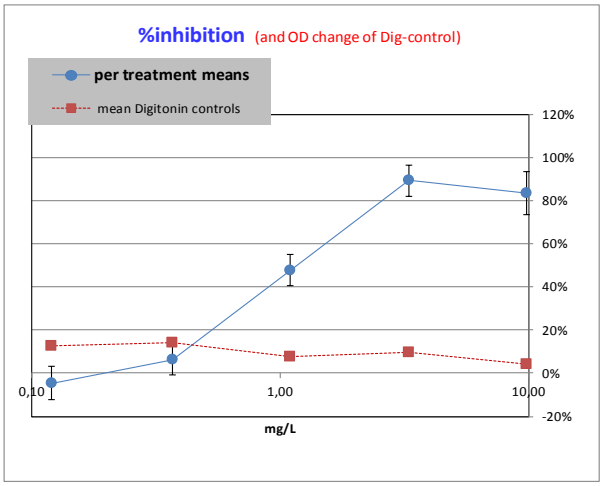
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,49	1,24	0,25
$C0_b$+	control1	1,47	1,3	0,17
C1+ in test[1]	9,86	1,54	1,44	0,1
C2+ in test[1]	3,29	1,51	1,37	0,14
C3+ in test[1]	1,1	1,49	1,37	0,12
C4+ in test[1]	0,37	1,49	1,26	0,23
C5+ in test[1]	0,12	1,5	1,31	0,19
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,52	1,36	0,16
$C0_b$+	control1	1,51	1,38	0,13
C1+ in test[1]	9,86	1,47	1,47	0
C2+ in test[1]	3,29	1,51	1,40	0,11
C3+ in test[1]	1,1	1,53	1,39	0,14
C4+ in test[1]	0,37	1,51	1,34	0,17
C5+ in test[1]	0,12	1,52	1,34	0,18
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



hexachlorophene: Lab_I_I (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,382	0,565	0,817	0,103	0,011	6	**0,618**	**0,115**	**0%**	19%	✓ 45%
	mean C1	9,86	1,523	1,353	0,170	0,020	0,0004	3	**0,103**	**0,061**	**83%**	10%	7%
	mean C2	3,29	1,477	1,263	0,213	0,021	0,0004	3	**0,067**	**0,045**	**89%**	7%	5%
	mean C3	1,10	1,430	0,997	0,433	0,025	0,0006	3	**0,323**	**0,044**	**48%**	7%	23%
	mean C4	0,37	1,390	0,597	0,793	0,021	0,0004	3	**0,580**	**0,043**	**6%**	7%	42%
	mean C5	0,12	1,380	0,547	0,833	0,047	0,0022	3	**0,647**	**0,048**	**-5%**	8%	47%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,500	1,302	0,198	0,0512	0,0026	6					✓ 13%
	mean C1+[1]	9,86	1,513	1,447	0,067	0,058	0,0033333	3					✓ 4%
	mean C2+[1]	3,29	1,520	1,373	0,147	0,040	0,0016333	3					✓ 10%
	mean C3+[1]	1,10	1,483	1,373	0,110	0,036	0,0013	3					✓ 7%
	mean C4+[1]	0,37	1,503	1,290	0,213	0,038	0,0014333	3					✓ 14%
	mean C5+[1]	0,12	1,500	1,313	0,187	0,006	3,333E-05	3					✓ 12%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

hexachlorophene: laboratory 2. test run 1 (Lab_2_1)

AS sampling date: 14-Jan-13
dry weight: 3,72 g/L

test date: 16-Jan-13
test substance: Hexachlorophen

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2	.	C2+
	C3	.	C3+
	C4	.	C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

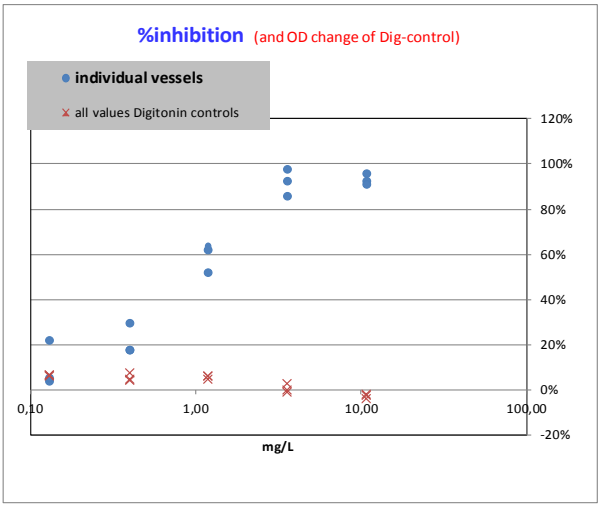
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,31	0,58	0,73
$C0_b$	control	1,36	0,67	0,69
C1 in test	10,67	1,54	1,54	0
C2 in test	3,56	1,50	1,41	0,09
C3 in test	1,19	1,41	1,11	0,3
C4 in test	0,40	1,35	0,85	0,5
C5 in test	0,13	1,36	0,80	0,56
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,34	0,65	0,69
$C0_b$	control	1,38	0,67	0,71
C1 in test	10,67	1,5	1,52	-0,02
C2 in test	3,56	1,46	1,41	0,05
C3 in test	1,19	1,43	1,06	0,37
C4 in test	0,40	1,4	0,83	0,57
C5 in test	0,13	1,37	0,7	0,67
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,40	0,75	0,65
$C0_b$	control	1,34	0,64	0,7
C1 in test	10,67	1,56	1,55	0,01
C2 in test	3,56	1,45	1,43	0,02
C3 in test	1,19	1,44	1,13	0,31
C4 in test	0,40	1,41	0,84	0,57
C5 in test	0,13	1,37	0,71	0,66
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,50	1,37	0,13
$C0_b$+	control1	1,53	1,42	0,11
C1+ in test[1]	10,67	1,56	1,62	-0,06
C2+ in test[1]	3,56	1,5	1,52	-0,02
C3+ in test[1]	1,19	1,53	1,44	0,09
C4+ in test[1]	0,40	1,49	1,42	0,07
C5+ in test[1]	0,13	1,53	1,44	0,09
C6+ in test[1]				
C7+ in test[1]				

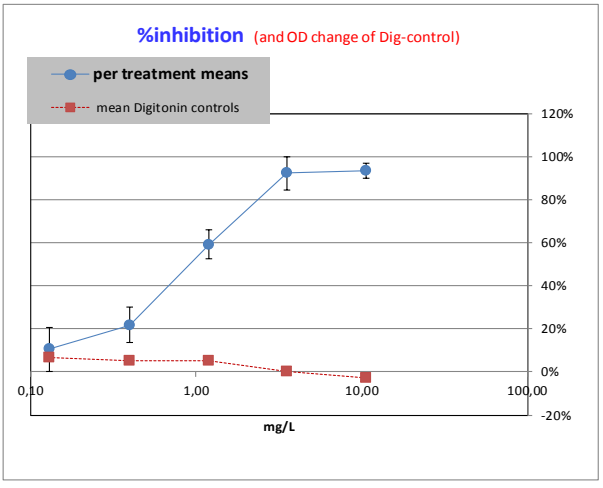
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,51	1,42	0,09
$C0_b$+	control1	1,49	1,43	0,06
C1+ in test[1]	10,67	1,6	1,63	-0,03
C2+ in test[1]	3,56	1,53	1,53	0
C3+ in test[1]	1,19	1,54	1,45	0,09
C4+ in test[1]	0,4	1,54	1,43	0,11
C5+ in test[1]	0,13	1,49	1,39	0,1
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,50	1,38	0,12
$C0_b$+	control1	1,48	1,40	0,08
C1+ in test[1]	10,67	1,61	1,65	-0,04
C2+ in test[1]	3,56	1,58	1,54	0,04
C3+ in test[1]	1,19	1,55	1,48	0,07
C4+ in test[1]	0,40	1,49	1,43	0,06
C5+ in test[1]	0,13	1,50	1,40	0,1
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



hexachlorophene: Lab_2_I (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,355	0,660	0,695	0,027	0,001	6	**0,597**	**0,038**	**0%**	6%	✓ 44%
mean C1	10,67	1,533	1,537	-0,003	0,015	0,0002	3	**0,040**	**0,022**	**93%**	4%	3%
mean C2	3,56	1,470	1,417	0,053	0,035	0,0012	3	**0,047**	**0,047**	**92%**	8%	3%
mean C3	1,19	1,427	1,100	0,327	0,038	0,0014	3	**0,243**	**0,040**	**59%**	7%	17%
mean C4	0,40	1,387	0,840	0,547	0,040	0,0016	3	**0,467**	**0,048**	**22%**	8%	34%
mean C5	0,13	1,367	0,737	0,630	0,061	0,0037	3	**0,533**	**0,061**	**11%**	10%	39%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
mean C7	#NV										0%	

Replicate2 1-3

mean $C0_b$+	0[1]	1,502	1,403	0,098	0,0264	0,0007	6					✓ 7%
mean C1+[1]	10,67	1,590	1,633	-0,043	0,015	0,0002333	3					✓ -3%
mean C2+[1]	3,56	1,537	1,530	0,007	0,031	0,0009333	3					✓ 0%
mean C3+[1]	1,19	1,540	1,457	0,083	0,012	0,0001333	3					✓ 5%
mean C4+[1]	0,40	1,507	1,427	0,080	0,026	0,0007	3					✓ 5%
mean C5+[1]	0,13	1,507	1,410	0,097	0,006	3,333E-05	3					✓ 6%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

hexachlorophene: laboratory 3. test run 1 (Lab_3_1)

AS sampling date 19-Feb-13
dry weight 5,29 g/L

test date 19-Feb-13
test substance hexachlorophen

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

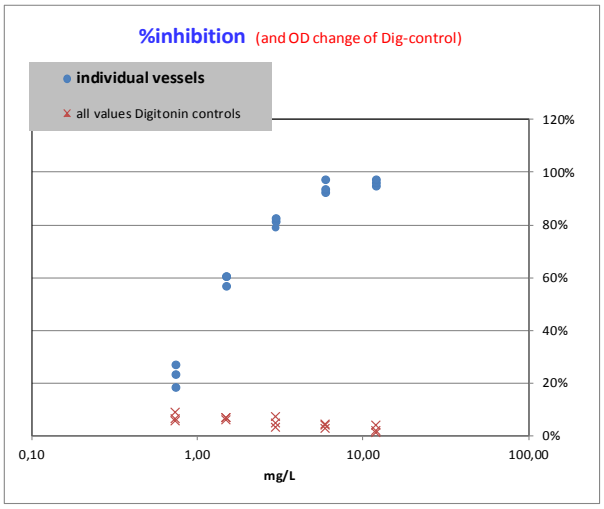
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,51	0,53	0,98
$C0_b$	control	1,49	0,56	0,93
C1 in test	11,86	1,62	1,54	0,08
C2 in test	5,93	1,59	1,47	0,12
C3 in test	2,97	1,55	1,30	0,25
C4 in test	1,48	1,56	1,11	0,45
C5 in test	0,74	1,49	0,76	0,73
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,51	0,62	0,89
$C0_b$	control	1,43	0,56	0,87
C1 in test	11,86	1,63	1,56	0,07
C2 in test	5,93	1,55	1,44	0,11
C3 in test	2,97	1,54	1,31	0,23
C4 in test	1,48	1,5	1,08	0,42
C5 in test	0,74	1,37	0,6	0,77
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,49	0,53	0,96
$C0_b$	control	1,31	0,48	0,83
C1 in test	11,86	1,64	1,58	0,06
C2 in test	5,93	1,50	1,42	0,08
C3 in test	2,97	1,51	1,29	0,22
C4 in test	1,48	1,48	1,06	0,42
C5 in test	0,74	1,43	0,73	0,7
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,52	1,41	0,11
$C0_b$+	control1	1,57	1,47	0,1
C1+ in test[1]	11,86	1,68	1,65	0,03
C2+ in test[1]	5,93	1,66	1,59	0,07
C3+ in test[1]	2,97	1,62	1,51	0,11
C4+ in test[1]	1,48	1,52	1,42	0,1
C5+ in test[1]	0,74	1,57	1,44	0,13
C6+ in test[1]				
C7+ in test[1]				

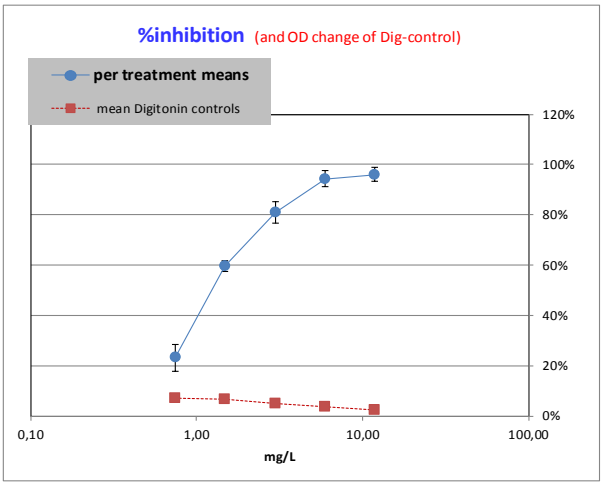
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,42	1,31	0,11
$C0_b$+	control1	1,34	1,28	0,06
C1+ in test[1]	11,86	1,46	1,4	0,06
C2+ in test[1]	5,93	1,43	1,39	0,04
C3+ in test[1]	2,97	1,46	1,39	0,07
C4+ in test[1]	1,48	1,43	1,34	0,09
C5+ in test[1]	0,74	1,39	1,3	0,09
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,37	1,32	0,05
$C0_b$+	control1	1,39	1,30	0,09
C1+ in test[1]	11,86	1,52	1,50	0,02
C2+ in test[1]	5,93	1,49	1,43	0,06
C3+ in test[1]	2,97	1,42	1,37	0,05
C4+ in test[1]	1,48	1,39	1,29	0,1
C5+ in test[1]	0,74	1,35	1,27	0,08
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



hexachlorophene: Lab_3_I (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
mean C0	0	1,457	0,547	0,910	0,057	0,003	6	**0,823**	**0,063**	**0%**	8%	✓ 57%
mean C1	11,86	1,630	1,560	0,070	0,010	0,0001	3	**0,033**	**0,023**	**96%**	3%	2%
mean C2	5,93	1,547	1,443	0,103	0,021	0,0004	3	**0,047**	**0,026**	**94%**	3%	3%
mean C3	2,97	1,533	1,300	0,233	0,015	0,0002	3	**0,157**	**0,034**	**81%**	4%	10%
mean C4	1,48	1,513	1,083	0,430	0,017	0,0003	3	**0,333**	**0,018**	**60%**	2%	22%
mean C5	0,74	1,430	0,697	0,733	0,035	0,0012	3	**0,633**	**0,044**	**23%**	5%	44%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!
mean C7	#NV										0%	

Replicate2 1-3

mean $C0_b$+	0[1]	1,435	1,348	0,087	0,0258	0,0007	6					✓ 6%
mean C1+[1]	11,86	1,553	1,517	0,037	0,021	0,0004333	3					✓ 2%
mean C2+[1]	5,93	1,527	1,470	0,057	0,015	0,0002333	3					✓ 4%
mean C3+[1]	2,97	1,500	1,423	0,077	0,031	0,0009333	3					✓ 5%
mean C4+[1]	1,48	1,447	1,350	0,097	0,006	3,333E-05	3					✓ 7%
mean C5+[1]	0,74	1,437	1,337	0,100	0,026	0,0007	3					✓ 7%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

hexachlorophene: laboratory 4. test run 1 (Lab_4_1)

AS sampling date	31-Aug-12
dry weight	3,5 g/L
test date	02-Sep-12
test substance	hexachlorophen

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

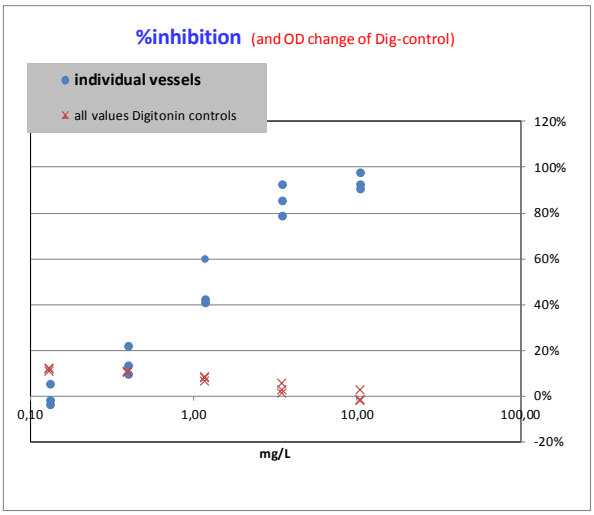
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,39	0,65	0,74
$C0_b$	control	1,41	0,69	0,72
C1 in test	10,41	1,41	1,37	0,04
C2 in test	3,47	1,38	1,29	0,09
C3 in test	1,16	1,40	1,06	0,34
C4 in test	0,39	1,41	0,81	0,6
C5 in test	0,13	1,37	0,61	0,76
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,42	0,69	0,73
$C0_b$	control	1,41	0,67	0,74
C1 in test	10,41	1,36	1,31	0,05
C2 in test	3,47	1,38	1,25	0,13
C3 in test	1,16	1,41	0,96	0,45
C4 in test	0,39	1,38	0,71	0,67
C5 in test	0,13	1,41	0,66	0,75
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,37	0,64	0,73
$C0_b$	control	1,38	0,66	0,72
C1 in test	10,41	1,39	1,38	0,01
C2 in test	3,47	1,40	1,23	0,17
C3 in test	1,16	1,38	0,94	0,44
C4 in test	0,39	1,37	0,72	0,65
C5 in test	0,13	1,36	0,65	0,71
C6 in test				
C7 in test				

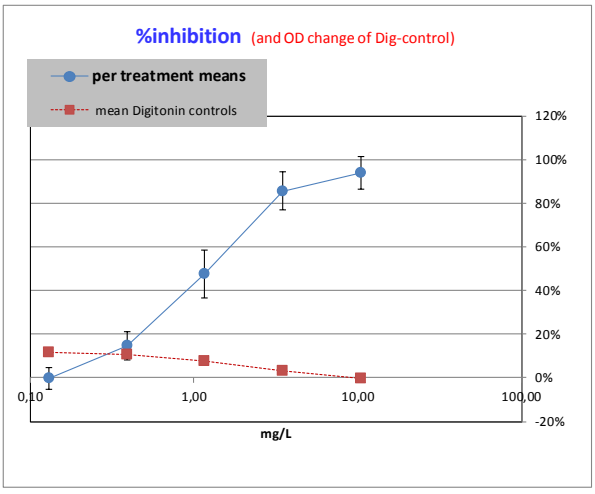


Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,39	1,25	0,14
$C0_b$+	control1	1,38	1,23	0,15
C1+ in test[1]	10,41	1,38	1,41	-0,03
C2+ in test[1]	3,47	1,37	1,35	0,02
C3+ in test[1]	1,16	1,4	1,31	0,09
C4+ in test[1]	0,39	1,39	1,25	0,14
C5+ in test[1]	0,13	1,37	1,21	0,16
C6+ in test[1]				
C7+ in test[1]				

Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,39	1,23	0,16
$C0_b$+	control1	1,41	1,26	0,15
C1+ in test[1]	10,41	1,36	1,32	0,04
C2+ in test[1]	3,47	1,39	1,31	0,08
C3+ in test[1]	1,16	1,4	1,29	0,11
C4+ in test[1]	0,39	1,36	1,21	0,15
C5+ in test[1]	0,13	1,38	1,23	0,15
C6+ in test[1]				
C7+ in test[1]				



Replicate 3 (+Digitonin)

$C0_a$+	control1	1,41	1,26	0,15
$C0_b$+	control1	1,40	1,24	0,16
C1+ in test[1]	10,41	1,39	1,41	-0,02
C2+ in test[1]	3,47	1,40	1,36	0,04
C3+ in test[1]	1,16	1,37	1,25	0,12
C4+ in test[1]	0,39	1,36	1,21	0,15
C5+ in test[1]	0,13	1,39	1,22	0,17
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin

hexachlorophene: Lab_4_I (continued)

	data analysis / evaluation											
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,397	0,667	0,730	0,009	0,000	6	**0,578**	**0,012**	**0%**	2%	✓ 41%
	mean C1	10,41	1,387	1,353	0,033	0,021	0,0004	3	**0,037**	**0,043**	**94%**	7%	3%
	mean C2	3,47	1,387	1,257	0,130	0,040	0,0016	3	**0,083**	**0,050**	**86%**	9%	6%
	mean C3	1,16	1,397	0,987	0,410	0,061	0,0037	3	**0,303**	**0,063**	**48%**	11%	22%
	mean C4	0,39	1,387	0,747	0,640	0,036	0,0013	3	**0,493**	**0,037**	**15%**	6%	36%
	mean C5	0,13	1,380	0,640	0,740	0,026	0,0007	3	**0,580**	**0,028**	**0%**	5%	42%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,397	1,245	0,152	0,0075	0,0001	6					✓ 11%
	mean C1+[1]	10,41	1,377	1,380	-0,003	0,038	0,0014333	3					✓ 0%
	mean C2+[1]	3,47	1,387	1,340	0,047	0,031	0,0009333	3					✓ 3%
	mean C3+[1]	1,16	1,390	1,283	0,107	0,015	0,0002333	3					✓ 8%
	mean C4+[1]	0,39	1,370	1,223	0,147	0,006	3,333E-05	3					✓ 11%
	mean C5+[1]	0,13	1,380	1,220	0,160	0,010	0,0001	3					✓ 12%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

hexachlorophene: laboratory 5. test run 1 (Lab_5_1)

AS sampling date	31-Jul-12
dry weight	4,8 g/L
test date	01-Aug-12
test substance	Hexachlorophen

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

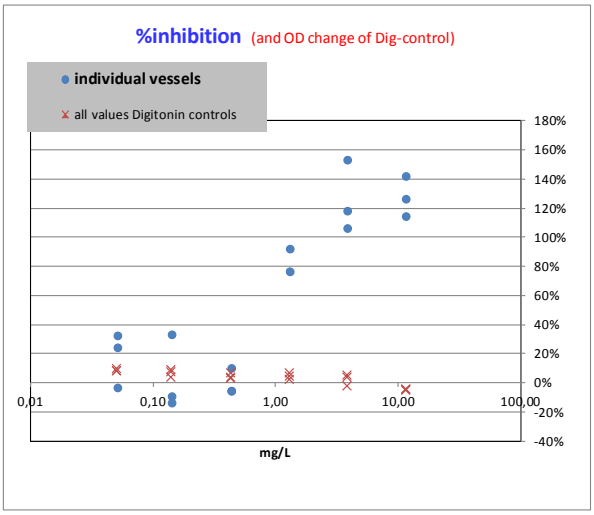
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,43	1,10	0,33
$C0_b$	control	1,45	1,12	0,33
C1 in test	11,58	1,46	1,63	-0,17
C2 in test	3,86	1,46	1,44	0,02
C3 in test	1,29	1,45	1,32	0,13
C4 in test	0,43	1,46	1,12	0,34
C5 in test	0,14	1,46	1,08	0,38
C6 in test	0,050	1,44	1,05	0,39
C7 in test				

Replicate 2

$C0_a$	control	1,45	1,09	0,36
$C0_b$	control	1,46	1,06	0,4
C1 in test	11,58	1,49	1,59	-0,1
C2 in test	3,86	1,41	1,42	-0,01
C3 in test	1,29	1,39	1,3	0,09
C4 in test	0,43	1,33	1,03	0,3
C5 in test	0,14	1,33	1,06	0,27
C6 in test	0,05	1,39	1,07	0,32
C7 in test				

Replicate 3

$C0_a$	control	1,42	1,07	0,35
$C0_b$	control	1,43	1,03	0,4
C1 in test	11,58	1,48	1,61	-0,13
C2 in test	3,86	1,44	1,54	-0,1
C3 in test	1,29	1,44	1,31	0,13
C4 in test	0,43	1,42	1,08	0,34
C5 in test	0,14	1,45	1,06	0,39
C6 in test	0,05	1,43	1,13	0,3
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,49	1,35	0,14
$C0_b$+	control1	1,48	1,36	0,12
C1+ in test[1]	11,58	1,49	1,56	-0,07
C2+ in test[1]	3,86	1,47	1,41	0,06
C3+ in test[1]	1,29	1,44	1,37	0,07
C4+ in test[1]	0,43	1,43	1,38	0,05
C5+ in test[1]	0,14	1,45	1,34	0,11
C6+ in test[1]	0,05	1,45	1,33	0,12
C7+ in test[1]				

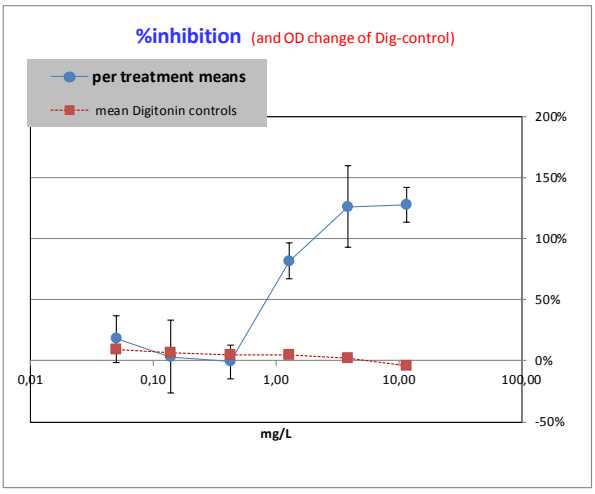
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,44	1,34	0,1
$C0_b$+	control1	1,42	1,32	0,1
C1+ in test[1]	11,58	1,49	1,55	-0,06
C2+ in test[1]	3,86	1,47	1,5	-0,03
C3+ in test[1]	1,29	1,46	1,36	0,1
C4+ in test[1]	0,43	1,43	1,33	0,1
C5+ in test[1]	0,14	1,44	1,38	0,06
C6+ in test[1]	0,05	1,40	1,28	0,12
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,44	1,36	0,08
$C0_b$+	control1	1,43	1,33	0,1
C1+ in test[1]	11,58	1,47	1,53	-0,06
C2+ in test[1]	3,86	1,48	1,40	0,08
C3+ in test[1]	1,29	1,44	1,40	0,04
C4+ in test[1]	0,43	1,42	1,36	0,06
C5+ in test[1]	0,14	1,45	1,32	0,13
C6+ in test[1]	0,05	1,48	1,34	0,14
C7+ in test[1]				

[1]) including Digitonin



hexachlorophene: Lab_5_I (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,440	1,078	0,362	0,032	0,001	6	**0,255**	**0,038**	**0%**	15%	✗ 18%
	mean C1	11,58	1,477	1,610	-0,133	0,035	0,0012	3	**-0,070**	**0,036**	**127%**	14%	-5%
	mean C2	3,86	1,437	1,467	-0,030	0,062	0,0039	3	**-0,067**	**0,086**	**126%**	34%	-5%
	mean C3	1,29	1,427	1,310	0,117	0,023	0,0005	3	**0,047**	**0,038**	**82%**	15%	3%
	mean C4	0,43	1,403	1,077	0,327	0,023	0,0005	3	**0,257**	**0,035**	**-1%**	14%	18%
	mean C5	0,14	1,413	1,067	0,347	0,067	0,0044	3	**0,247**	**0,076**	**3%**	30%	17%
	mean C6	0,050	1,420	1,083	0,337	0,047	0,0022	3	**0,210**	**0,049**	**18%**	19%	15%
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_h$+	0[1]	1,450	1,343	0,107	0,0207	0,0004	6					✓ 7%
	mean C1+[1]	11,58	1,483	1,547	-0,063	0,006	3,333E-05	3					✓ -4%
	mean C2+[1]	3,86	1,473	1,437	0,037	0,059	0,0034333	3					✓ 2%
	mean C3+[1]	1,29	1,447	1,377	0,070	0,030	0,0009	3					✓ 5%
	mean C4+[1]	0,43	1,427	1,357	0,070	0,026	0,0007	3					✓ 5%
	mean C5+[1]	0,14	1,447	1,347	0,100	0,036	0,0013	3					✓ 7%
	mean C6+[1]	0,05	1,443	1,317	0,127	0,012	0,000133333	3					✓ 9%
	mean C7+[1]	#NV											

[1]) including Digitonin

hexachlorophene: laboratory 5. test run 2 (Lab_5_2)

AS sampling date 04-Sep-12
dry weight 3,6 g/L

test date 06-Sep-12
test substance hexachlorophen

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2	.	C2+
	C3	.	C3+
	C4	.	C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

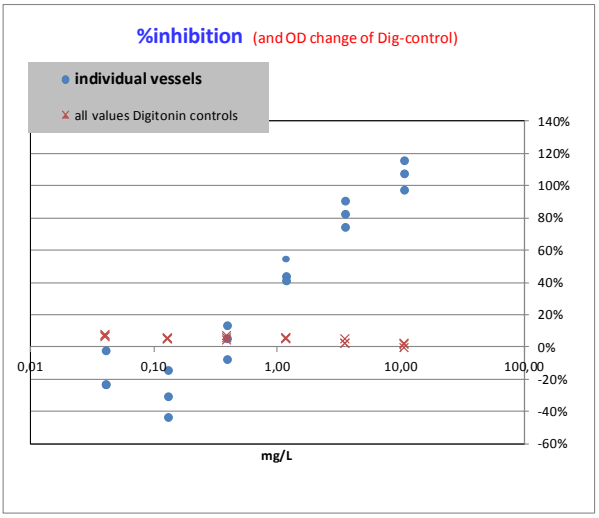
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,47	0,94	0,53
$C0_b$	control	1,40	0,91	0,49
C1 in test	10,53	1,48	1,52	-0,04
C2 in test	3,51	1,47	1,38	0,09
C3 in test	1,17	1,46	1,20	0,26
C4 in test	0,39	1,46	1,04	0,42
C5 in test	0,13	1,48	0,96	0,52
C6 in test	0,040	1,54	0,96	0,58
C7 in test				

Replicate 2

$C0_a$	control	1,41	0,98	0,43
$C0_b$	control	1,41	0,94	0,47
C1 in test	10,53	1,56	1,53	0,03
C2 in test	3,51	1,5	1,38	0,12
C3 in test	1,17	1,48	1,18	0,3
C4 in test	0,39	1,47	1,02	0,45
C5 in test	0,13	1,53	0,9	0,63
C6 in test	0,04	1,42	0,92	0,5
C7 in test				

Replicate 3

$C0_a$	control	1,42	0,98	0,44
$C0_b$	control	1,41	0,99	0,42
C1 in test	10,53	1,55	1,56	-0,01
C2 in test	3,51	1,53	1,38	0,15
C3 in test	1,17	1,47	1,16	0,31
C4 in test	0,39	1,45	0,95	0,5
C5 in test	0,13	1,47	0,89	0,58
C6 in test	0,04	1,44	0,86	0,58
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,51	1,42	0,09
$C0_b$+	control1	1,50	1,42	0,08
C1+ in test[1]	10,53	1,58	1,54	0,04
C2+ in test[1]	3,51	1,52	1,48	0,04
C3+ in test[1]	1,17	1,51	1,42	0,09
C4+ in test[1]	0,39	1,53	1,42	0,11
C5+ in test[1]	0,13	1,49	1,41	0,08
C6+ in test[1]	0,04	1,51	1,41	0,1
C7+ in test[1]				

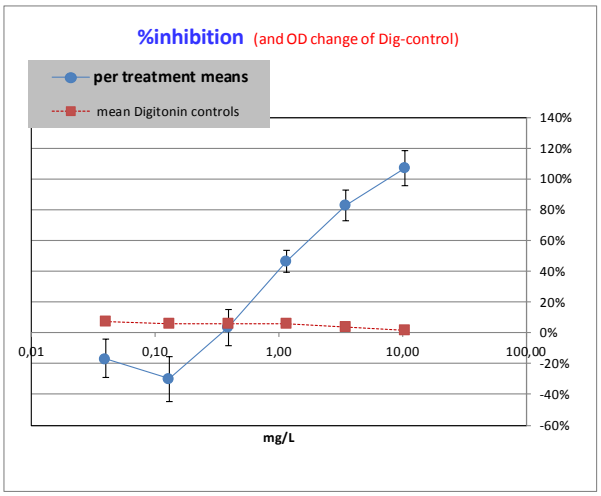
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,49	1,4	0,09
$C0_b$+	control1	1,47	1,42	0,05
C1+ in test[1]	10,53	1,58	1,55	0,03
C2+ in test[1]	3,51	1,52	1,44	0,08
C3+ in test[1]	1,17	1,5	1,42	0,08
C4+ in test[1]	0,39	1,56	1,49	0,07
C5+ in test[1]	0,13	1,5	1,42	0,08
C6+ in test[1]	0,04	1,50	1,39	0,11
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,49	1,38	0,11
$C0_b$+	control1	1,48	1,40	0,08
C1+ in test[1]	10,53	1,55	1,56	-0,01
C2+ in test[1]	3,51	1,51	1,47	0,04
C3+ in test[1]	1,17	1,52	1,43	0,09
C4+ in test[1]	0,39	1,50	1,41	0,09
C5+ in test[1]	0,13	1,50	1,41	0,09
C6+ in test[1]	0,04	1,52	1,40	0,12
C7+ in test[1]				

[1]) including Digitonin



hexachlorophene: Lab_5_2 (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,420	0,957	0,463	0,042	0,002	6	**0,380**	**0,046**	**0%**	12%	27%
	mean C1	10,53	1,530	1,537	-0,007	0,035	0,0012	3	**-0,027**	**0,044**	**107%**	12%	-2%
	mean C2	3,51	1,500	1,380	0,120	0,030	0,0009	3	**0,067**	**0,038**	**82%**	10%	4%
	mean C3	1,17	1,470	1,180	0,290	0,026	0,0007	3	**0,203**	**0,027**	**46%**	7%	14%
	mean C4	0,39	1,460	1,003	0,457	0,040	0,0016	3	**0,367**	**0,045**	**4%**	12%	25%
	mean C5	0,13	1,493	0,917	0,577	0,055	0,0030	3	**0,493**	**0,055**	**-30%**	15%	33%
	mean C6	0,040	1,467	0,913	0,553	0,046	0,0021	3	**0,443**	**0,047**	-17%	12%	30%
	mean C7	#NV										0%	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,490	1,407	0,083	0,0197	0,0004	6					✓ 6%
	mean C1+[1]	10,53	1,570	1,550	0,020	0,026	0,0007	3					✓ 1%
	mean C2+[1]	3,51	1,517	1,463	0,053	0,023	0,0005333	3					✓ 4%
	mean C3+[1]	1,17	1,510	1,423	0,087	0,006	3,333E-05	3					✓ 6%
	mean C4+[1]	0,39	1,530	1,440	0,090	0,020	0,0004	3					✓ 6%
	mean C5+[1]	0,13	1,497	1,413	0,083	0,006	3,333E-05	3					✓ 6%
	mean C6+[1]	0,04	1,510	1,400	0,110	0,010	0,0001	3					✓ 7%
	mean C7+[1]	#NV											

[1]) including Digitonin

phenyl ether: laboratory I. test run I (Lab_I_I)

AS sampling date: 10-Apr-13
dry weight: 3,155 g/L

test date: 11-Apr-13
test substance: phenyl ether

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

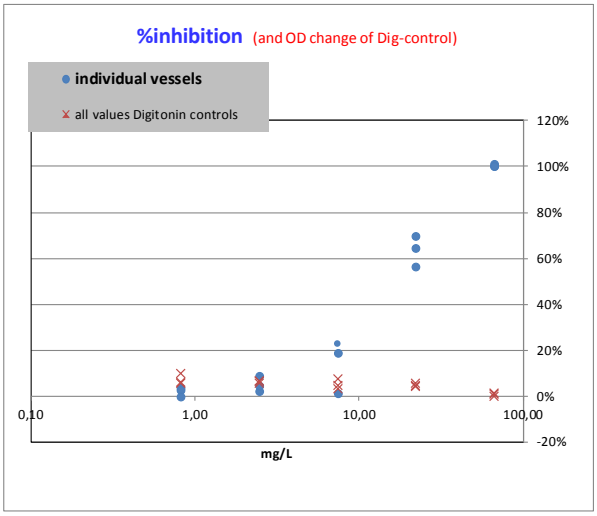
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,26	0,45	0,81
$C0_b$	control	1,25	0,47	0,78
C1 in test	66,30	1,47	1,47	0
C2 in test	22,10	1,38	1,09	0,29
C3 in test	7,37	1,34	0,70	0,64
C4 in test	2,46	1,30	0,54	0,76
C5 in test	0,82	1,25	0,44	0,81
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,46	0,47	0,99
$C0_b$	control	1,26	0,45	0,81
C1 in test	66,30	1,44	1,43	0,01
C2 in test	22,10	1,34	1,01	0,33
C3 in test	7,37	1,45	0,65	0,8
C4 in test	2,46	1,31	0,5	0,81
C5 in test	0,82	1,27	0,45	0,82
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,32	0,47	0,85
$C0_b$	control	1,27	0,45	0,82
C1 in test	66,30	1,51	1,50	0,01
C2 in test	22,10	1,36	0,97	0,39
C3 in test	7,37	1,31	0,64	0,67
C4 in test	2,46	1,31	0,52	0,79
C5 in test	0,82	1,29	0,45	0,84
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,52	1,46	0,06
$C0_b$+	control1	1,47	1,39	0,08
C1+ in test[1]	66,30	1,55	1,54	0,01
C2+ in test[1]	22,1	1,48	1,41	0,07
C3+ in test[1]	7,37	1,54	1,43	0,11
C4+ in test[1]	2,46	1,51	1,43	0,08
C5+ in test[1]	0,82	1,49	1,4	0,09
C6+ in test[1]				
C7+ in test[1]				

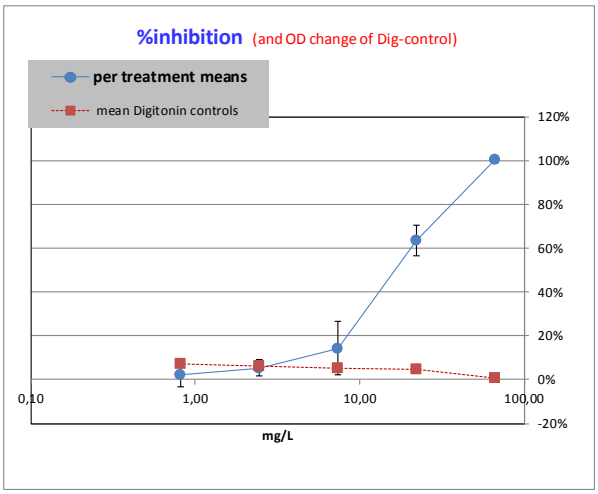
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,51	1,33	0,18
$C0_b$+	control1	1,61	1,46	0,15
C1+ in test[1]	66,30	1,55	1,55	0
C2+ in test[1]	22,1	1,5	1,42	0,08
C3+ in test[1]	7,37	1,48	1,41	0,07
C4+ in test[1]	2,46	1,46	1,36	0,1
C5+ in test[1]	0,82	1,49	1,34	0,15
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,49	1,41	0,08
$C0_b$+	control1	1,59	1,47	0,12
C1+ in test[1]	66,30	1,51	1,49	0,02
C2+ in test[1]	22,1	1,49	1,43	0,06
C3+ in test[1]	7,37	1,48	1,43	0,05
C4+ in test[1]	2,46	1,51	1,41	0,1
C5+ in test[1]	0,82	1,49	1,41	0,08
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



phenyl ether: Lab_1_1 (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	**mean ΔOD_{corr}**	**StDev**	**% inhibition**	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,303	0,460	0,843	0,075	0,006	6	**0,732**	**0,089**	**0%**	12%	✓ 56%
	mean C1	66,30	1,473	1,467	0,007	0,006	0,0000	3	**-0,003**	**0,012**	**100%**	2%	0%
	mean C2	22,10	1,360	1,023	0,337	0,050	0,0025	3	**0,267**	**0,051**	**64%**	7%	20%
	mean C3	7,37	1,367	0,663	0,703	0,085	0,0072	3	**0,627**	**0,090**	**14%**	12%	46%
	mean C4	2,46	1,307	0,520	0,787	0,025	0,0006	3	**0,693**	**0,028**	**5%**	4%	53%
	mean C5	0,82	1,270	0,447	0,823	0,015	0,0002	3	**0,717**	**0,041**	**2%**	6%	56%
	mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,532	1,420	0,112	0,0467	0,0022	6					✓ 7%
	mean C1+[1]	66,30	1,537	1,527	0,010	0,010	0,0001	3					✓ 1%
	mean C2+[1]	22,10	1,490	1,420	0,070	0,010	0,0001	3					✓ 5%
	mean C3+[1]	7,37	1,500	1,423	0,077	0,031	0,0009333	3					✓ 5%
	mean C4+[1]	2,46	1,493	1,400	0,093	0,012	0,0001333	3					✓ 6%
	mean C5+[1]	0,82	1,490	1,383	0,107	0,038	0,0014333	3					✓ 7%
	mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
	mean C7+[1]	#NV											

[1]) including Digitonin

phenyl ether: laboratory 2. test run I (Lab_2_I)

AS sampling date: 18-Jan-13
dry weight: 2,09 g/L

test date: 16-Jan-13
test substance: Phenylether

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

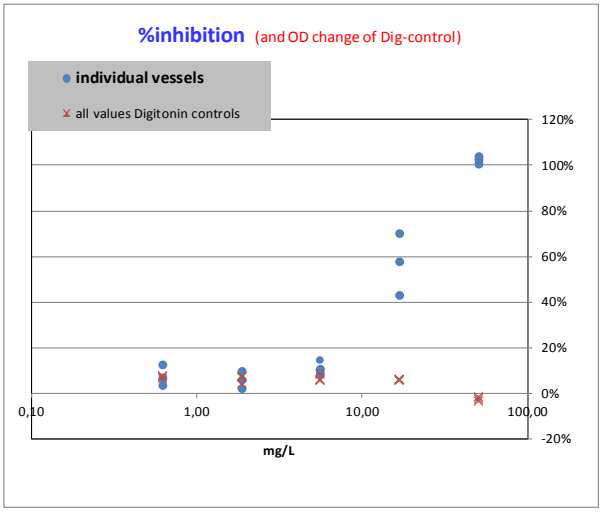
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,29	0,66	0,63
$C0_b$	control	1,35	0,68	0,67
C1 in test	50,15	1,52	1,56	-0,04
C2 in test	16,72	1,40	1,15	0,25
C3 in test	5,57	1,35	0,78	0,57
C4 in test	1,86	1,33	0,74	0,59
C5 in test	0,62	1,33	0,69	0,64
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,31	0,66	0,65
$C0_b$	control	1,34	0,66	0,68
C1 in test	50,15	1,46	1,52	-0,06
C2 in test	16,72	1,36	0,96	0,4
C3 in test	5,57	1,36	0,76	0,6
C4 in test	1,86	1,33	0,7	0,63
C5 in test	0,62	1,36	0,77	0,59
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,38	0,74	0,64
$C0_b$	control	1,30	0,66	0,64
C1 in test	50,15	1,48	1,53	-0,05
C2 in test	16,72	1,38	1,06	0,32
C3 in test	5,57	1,34	0,75	0,59
C4 in test	1,86	1,36	0,75	0,61
C5 in test	0,62	1,34	0,72	0,62
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,47	1,36	0,11
$C0_b$+	control1	1,45	1,36	0,09
C1+ in test[1]	50,15	1,5	1,55	-0,05
C2+ in test[1]	16,72	1,5	1,42	0,08
C3+ in test[1]	5,57	1,46	1,37	0,09
C4+ in test[1]	1,86	1,47	1,37	0,1
C5+ in test[1]	0,62	1,52	1,41	0,11
C6+ in test[1]				
C7+ in test[1]				

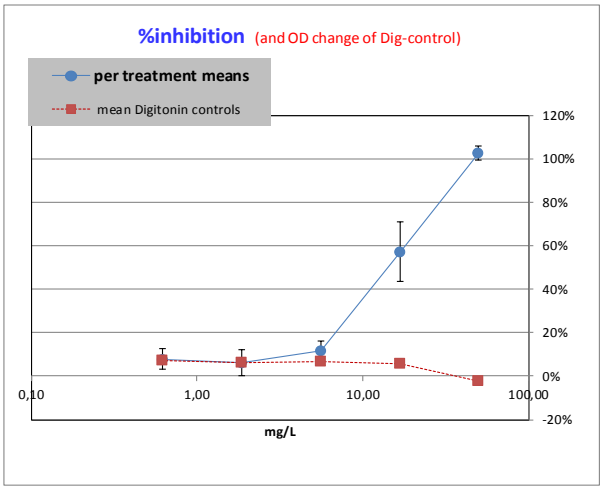
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,45	1,36	0,09
$C0_b$+	control1	1,47	1,38	0,09
C1+ in test[1]	50,15	1,53	1,55	-0,02
C2+ in test[1]	16,72	1,49	1,4	0,09
C3+ in test[1]	5,57	1,46	1,34	0,12
C4+ in test[1]	1,86	1,45	1,39	0,06
C5+ in test[1]	0,62	1,46	1,36	0,1
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,51	1,39	0,12
$C0_b$+	control1	1,45	1,36	0,09
C1+ in test[1]	50,15	1,45	1,49	-0,04
C2+ in test[1]	16,72	1,49	1,40	0,09
C3+ in test[1]	5,57	1,46	1,38	0,08
C4+ in test[1]	1,86	1,49	1,38	0,11
C5+ in test[1]	0,62	1,46	1,35	0,11
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



phenyl ether: Lab_2_1 (continued)

data analysis / evaluation

Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,328	0,677	0,652	0,019	0,000	6	**0,553**	**0,024**	**0%**	4%	✓ 42%
mean C1	50,15	1,487	1,537	-0,050	0,010	0,0001	3	**-0,013**	**0,018**	**102%**	3%	-1%
mean C2	16,72	1,380	1,057	0,323	0,075	0,0056	3	**0,237**	**0,075**	**57%**	14%	17%
mean C3	5,57	1,350	0,763	0,587	0,015	0,0002	3	**0,490**	**0,026**	**11%**	5%	36%
mean C4	1,86	1,340	0,730	0,610	0,020	0,0004	3	**0,520**	**0,033**	**6%**	6%	39%
mean C5	0,62	1,343	0,727	0,617	0,025	0,0006	3	**0,510**	**0,026**	**8%**	5%	38%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!
mean C7	#NV										0%	

Replicate2 1-3

mean $C0_b$+	0[1]	1,467	1,368	0,098	0,0133	0,0002	6					✓ 7%
mean C1+[1]	50,15	1,493	1,530	-0,037	0,015	0,0002333	3					✓ -2%
mean C2+[1]	16,72	1,493	1,407	0,087	0,006	3,333E-05	3					✓ 6%
mean C3+[1]	5,57	1,460	1,363	0,097	0,021	0,0004333	3					✓ 7%
mean C4+[1]	1,86	1,470	1,380	0,090	0,026	0,0007	3					✓ 6%
mean C5+[1]	0,62	1,480	1,373	0,107	0,006	3,333E-05	3					✓ 7%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

phenyl ether: laboratory 3. test run I (Lab_3_I)

AS sampling date: 14-Feb-13
dry weight: 5,32 g/L

test date: 14-Feb-13
test substance: phenyl ether

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

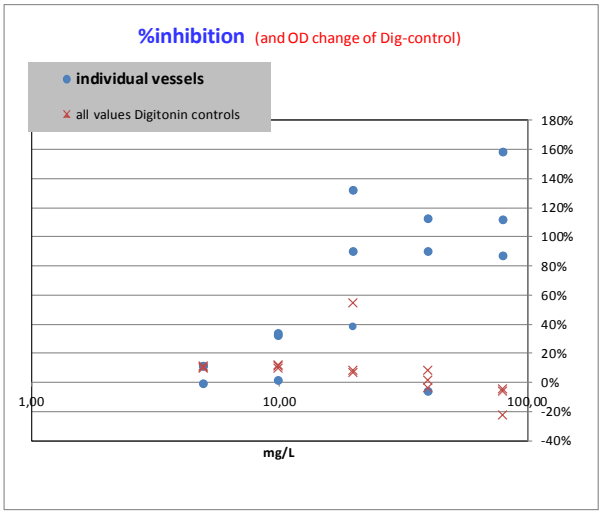
Code	mg/L	2h-OD_{440}	22h-OD_{440}	ΔOD
$C0_a$	control	1,09	0,22	0,87
$C0_b$	control	1,08	0,26	0,82
C1 in test	79,20	1,42	1,96	-0,54
C2 in test	39,60	1,16	0,44	0,72
C3 in test	19,80	1,21	0,47	0,74
C4 in test	9,90	1,21	0,41	0,8
C5 in test	4,95	1,11	0,29	0,82
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,07	0,21	0,86
$C0_b$	control	1,12	0,26	0,86
C1 in test	79,20	1,38	1,45	-0,07
C2 in test	39,60	1,3	1,21	0,09
C3 in test	19,80	1,19	0,79	0,4
C4 in test	9,90	1,09	0,49	0,6
C5 in test	4,95	1,11	0,37	0,74
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,08	0,25	0,83
$C0_b$	control	1,17	0,31	0,86
C1 in test	79,20	1,34	1,57	-0,23
C2 in test	39,60	1,39	1,45	-0,06
C3 in test	19,80	1,24	1,12	0,12
C4 in test	9,90	0,97	0,38	0,59
C5 in test	4,95	1,30	0,56	0,74
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,73	1,58	0,15
$C0_b$+	control1	1,25	1,04	0,21
C1+ in test[1]	79,20	1,3	1,38	-0,08
C2+ in test[1]	39,6	1,46	1,44	0,02
C3+ in test[1]	19,8	1,34	1,22	0,12
C4+ in test[1]	9,90	1,55	1,38	0,17
C5+ in test[1]	4,95	1,78	1,63	0,15
C6+ in test[1]				
C7+ in test[1]				

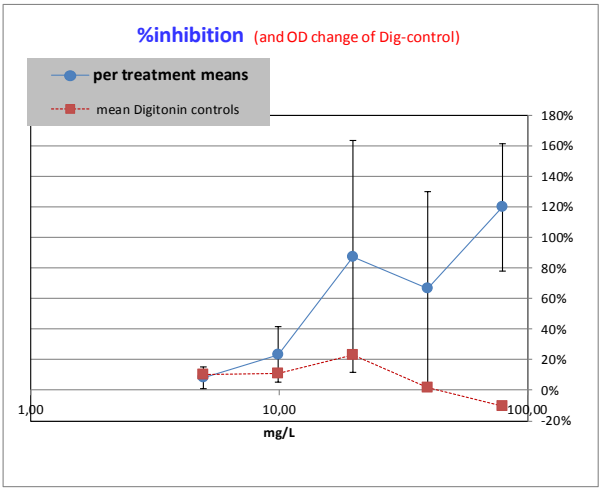
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,44	1,25	0,19
$C0_b$+	control1	1,18	1,02	0,16
C1+ in test[1]	79,20	1,45	1,51	-0,06
C2+ in test[1]	39,6	1,22	1,27	-0,05
C3+ in test[1]	19,8	1,19	1,09	0,1
C4+ in test[1]	9,9	1,17	1,01	0,16
C5+ in test[1]	4,95	1,42	1,25	0,17
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,35	1,16	0,19
$C0_b$+	control1	1,37	1,14	0,23
C1+ in test[1]	79,20	1,44	1,75	-0,31
C2+ in test[1]	39,6	1,29	1,18	0,11
C3+ in test[1]	19,8	1,80	1,01	0,79
C4+ in test[1]	9,90	1,48	1,34	0,14
C5+ in test[1]	4,95	1,38	1,22	0,16
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



phenyl ether: Lab_3_1 (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,102	0,252	0,850	0,020	0,000	6	**0,662**	**0,036**	**0%**	5%	✓ 60%
mean C1	79,20	1,380	1,660	-0,280	0,239	0,0571	3	**-0,130**	**0,276**	**120%**	42%	-9%
mean C2	39,60	1,283	1,033	0,250	0,414	0,1713	3	**0,223**	**0,422**	**66%**	64%	17%
mean C3	19,80	1,213	0,793	0,420	0,310	0,0964	3	**0,083**	**0,501**	**87%**	76%	7%
mean C4	9,90	1,090	0,427	0,663	0,118	0,0140	3	**0,507**	**0,119**	**23%**	18%	46%
mean C5	4,95	1,173	0,407	0,767	0,046	0,0021	3	**0,607**	**0,047**	**8%**	7%	52%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
mean C7	#NV										**0%**	

Replicate2 1-3

mean $C0_b$+	0[1]	1,387	1,198	0,188	0,0299	0,0009	6					✓ 14%
mean C1+[1]	79,20	1,397	1,547	-0,150	0,139	0,0193	3					✗ -11%
mean C2+[1]	39,60	1,323	1,297	0,027	0,080	0,0064333	3					✓ 2%
mean C3+[1]	19,80	1,443	1,107	0,337	0,393	0,1542333	3					✓ 23%
mean C4+[1]	9,90	1,400	1,243	0,157	0,015	0,0002333	3					✓ 11%
mean C5+[1]	4,95	1,527	1,367	0,160	0,010	1E-04	3					✓ 10%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

phenyl ether: laboratory 4. test run I (Lab_4_I)

AS sampling date: 23-Nov-12
dry weight: 4,9 g/L

test date: 23-Nov-12
test substance: pheny ether

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

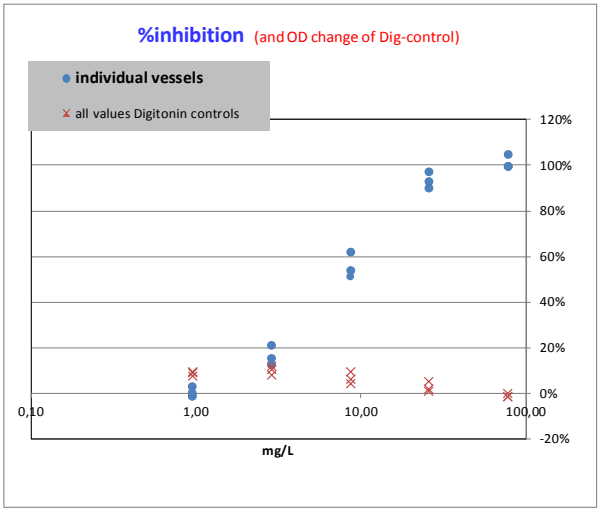
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,45	0,58	0,87
$C0_b$	control	1,47	0,61	0,86
C1 in test	77,59	1,51	1,56	-0,05
C2 in test	25,86	1,49	1,38	0,11
C3 in test	8,62	1,46	1,01	0,45
C4 in test	2,87	1,47	0,75	0,72
C5 in test	0,96	1,45	0,62	0,83
C6 in test				
C7 in test				

Replicate 2

$C0_a$	control	1,49	0,65	0,84
$C0_b$	control	1,46	0,59	0,87
C1 in test	77,59	1,5	1,51	-0,01
C2 in test	25,86	1,48	1,39	0,09
C3 in test	8,62	1,46	1,09	0,37
C4 in test	2,87	1,47	0,69	0,78
C5 in test	0,96	1,45	0,59	0,86
C6 in test				
C7 in test				

Replicate 3

$C0_a$	control	1,47	0,58	0,89
$C0_b$	control	1,46	0,62	0,84
C1 in test	77,59	1,52	1,53	-0,01
C2 in test	25,86	1,48	1,42	0,06
C3 in test	8,62	1,46	1,03	0,43
C4 in test	2,87	1,45	0,69	0,76
C5 in test	0,96	1,46	0,61	0,85
C6 in test				
C7 in test				



Replicate 1 (+Digitonin)

Code	mg/L	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,50	1,36	0,14
$C0_b$+	control1	1,48	1,35	0,13
C1+ in test[1]	77,59	1,51	1,53	-0,02
C2+ in test[1]	25,86	1,52	1,49	0,03
C3+ in test[1]	8,62	1,49	1,35	0,14
C4+ in test[1]	2,87	1,48	1,33	0,15
C5+ in test[1]	0,96	1,5	1,37	0,13
C6+ in test[1]				
C7+ in test[1]				

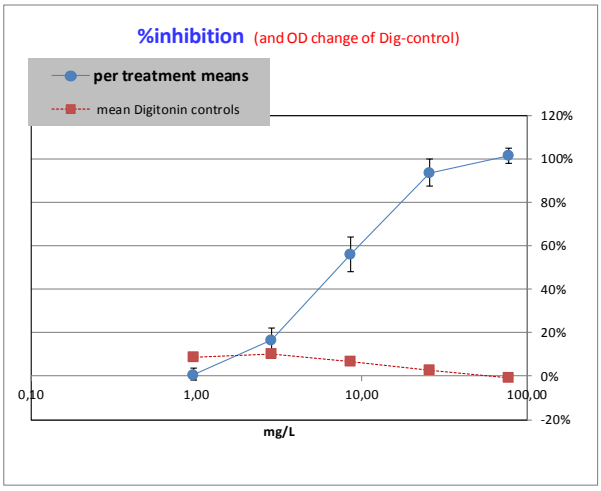
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,46	1,32	0,14
$C0_b$+	control1	1,47	1,34	0,13
C1+ in test[1]	77,59	1,5	1,5	0
C2+ in test[1]	25,86	1,49	1,48	0,01
C3+ in test[1]	8,62	1,46	1,37	0,09
C4+ in test[1]	2,87	1,47	1,35	0,12
C5+ in test[1]	0,96	1,45	1,31	0,14
C6+ in test[1]				
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,46	1,32	0,14
$C0_b$+	control1	1,49	1,36	0,13
C1+ in test[1]	77,59	1,51	1,53	-0,02
C2+ in test[1]	25,86	1,49	1,41	0,08
C3+ in test[1]	8,62	1,45	1,39	0,06
C4+ in test[1]	2,87	1,46	1,29	0,17
C5+ in test[1]	0,96	1,42	1,31	0,11
C6+ in test[1]				
C7+ in test[1]				

[1]) including Digitonin



phenyl ether: Lab_4_I (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,467	0,605	0,862	0,019	0,000	6	**0,727**	**0,020**	**0%**	3%	✓ 50%
mean C1	77,59	1,510	1,533	-0,023	0,023	0,0005	3	**-0,010**	**0,026**	**101%**	4%	-1%
mean C2	25,86	1,483	1,397	0,087	0,025	0,0006	3	**0,047**	**0,044**	**94%**	6%	3%
mean C3	8,62	1,460	1,043	0,417	0,042	0,0017	3	**0,320**	**0,058**	**56%**	8%	22%
mean C4	2,87	1,463	0,710	0,753	0,031	0,0009	3	**0,607**	**0,040**	**17%**	5%	41%
mean C5	0,96	1,453	0,607	0,847	0,015	0,0002	3	**0,720**	**0,022**	**1%**	3%	50%
mean C6	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0	**#DIV/0!**	**#DIV/0!**		#DIV/0!	#DIV/0!
mean C7	#NV										**0%**	

Replicate2 1-3

mean $C0_b$+	0[1]	1,477	1,342	0,135	0,0055	0,0000	6					✓ 9%
mean C1+[1]	77,59	1,507	1,520	-0,013	0,012	0,0001333	3					✓ -1%
mean C2+[1]	25,86	1,500	1,460	0,040	0,036	0,0013	3					✓ 3%
mean C3+[1]	8,62	1,467	1,370	0,097	0,040	0,0016333	3					✓ 7%
mean C4+[1]	2,87	1,470	1,323	0,147	0,025	0,0006333	3					✓ 10%
mean C5+[1]	0,96	1,457	1,330	0,127	0,015	0,0002333	3					✓ 9%
mean C6+[1]	#NV	#DIV/0!	#DIV/0!	#DIV/0!	0,000	#DIV/0!	0					
mean C7+[1]	#NV											

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

phenyl ether: laboratory 5. test run I (Lab_5_I)

AS sampling date	24-Jul-12
dry weight	4,9 g/L
test date	26-Jul-12
test substance	phenyl ether

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
↓	C2	↓	C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

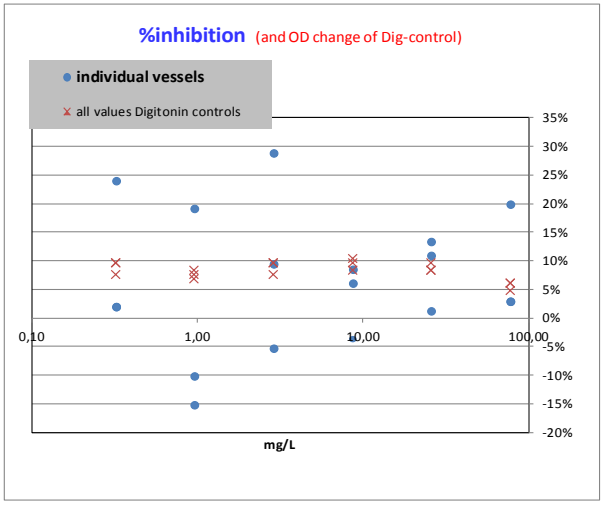
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,45	0,91	0,54
$C0_b$	control	1,44	0,94	0,5
C1 in test	77,59	1,44	0,96	0,48
C2 in test	25,86	1,45	0,96	0,49
C3 in test	8,62	1,43	0,87	0,56
C4 in test	2,87	1,43	0,87	0,56
C5 in test	0,96	1,46	0,88	0,58
C6 in test	0,320	1,43	0,90	0,53
C7 in test				

Replicate 2

$C0_a$	control	1,45	0,9	0,55
$C0_b$	control	1,48	0,92	0,56
C1 in test	77,59	1,41	1	0,41
C2 in test	25,86	1,46	0,93	0,53
C3 in test	8,62	1,44	0,92	0,52
C4 in test	2,87	1,44	0,94	0,5
C5 in test	0,96	1,47	0,91	0,56
C6 in test	0,32	1,36	0,92	0,44
C7 in test				

Replicate 3

$C0_a$	control	1,42	0,99	0,43
$C0_b$	control	1,43	0,89	0,54
C1 in test	77,59	1,45	0,97	0,48
C2 in test	25,86	1,44	0,96	0,48
C3 in test	8,62	1,43	0,92	0,51
C4 in test	2,87	1,38	0,96	0,42
C5 in test	0,96	1,42	0,98	0,44
C6 in test	0,32	1,41	0,88	0,53
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,44	1,33	0,11
$C0_b$+	control1	1,44	1,32	0,12
C1+ in test[1]	77,59	1,43	1,36	0,07
C2+ in test[1]	25,86	1,46	1,32	0,14
C3+ in test[1]	8,62	1,44	1,3	0,14
C4+ in test[1]	2,87	1,44	1,3	0,14
C5+ in test[1]	0,96	1,45	1,35	0,1
C6+ in test[1]	0,32	1,44	1,30	0,14
C7+ in test[1]				

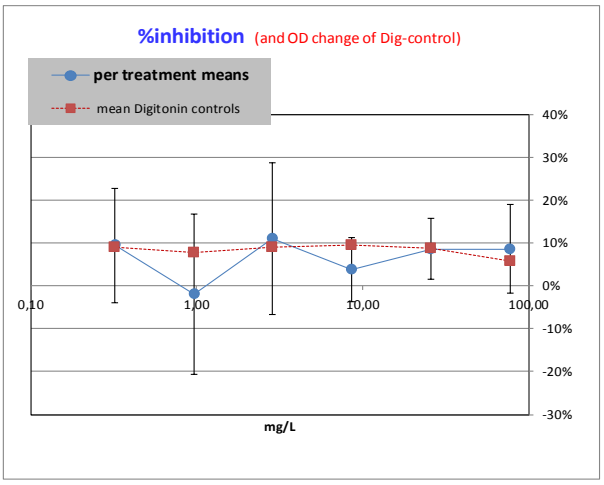
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,42	1,36	0,06
$C0_b$+	control1	1,48	1,32	0,16
C1+ in test[1]	77,59	1,45	1,36	0,09
C2+ in test[1]	25,86	1,46	1,34	0,12
C3+ in test[1]	8,62	1,45	1,3	0,15
C4+ in test[1]	2,87	1,43	1,32	0,11
C5+ in test[1]	0,96	1,45	1,33	0,12
C6+ in test[1]	0,32	1,47	1,33	0,14
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,41	1,30	0,11
$C0_b$+	control1	1,43	1,32	0,11
C1+ in test[1]	77,59	1,48	1,39	0,09
C2+ in test[1]	25,86	1,42	1,30	0,12
C3+ in test[1]	8,62	1,44	1,32	0,12
C4+ in test[1]	2,87	1,46	1,32	0,14
C5+ in test[1]	0,96	1,42	1,31	0,11
C6+ in test[1]	0,32	1,44	1,33	0,11
C7+ in test[1]				

[1]) including Digitonin



phenyl ether: Lab_5_1 (continued)

data analysis / evaluation												
Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
mean C0	0	1,445	0,925	0,520	0,049	0,002	6	**0,408**	**0,058**	**0%**	14%	✓ **28%**
mean C1	77,59	1,433	0,977	0,457	0,040	0,0016	3	**0,373**	**0,042**	**9%**	10%	26%
mean C2	25,86	1,450	0,950	0,500	0,026	0,0007	3	**0,373**	**0,029**	**9%**	7%	26%
mean C3	8,62	1,433	0,903	0,530	0,026	0,0007	3	**0,393**	**0,031**	**4%**	7%	27%
mean C4	2,87	1,417	0,923	0,493	0,070	0,0049	3	**0,363**	**0,072**	**11%**	18%	26%
mean C5	0,96	1,450	0,923	0,527	0,076	0,0057	3	**0,417**	**0,076**	**-2%**	19%	29%
mean C6	0,320	1,400	0,900	0,500	0,052	0,0027	3	**0,370**	**0,055**	**9%**	13%	26%
mean C7	#NV										0%	
mean $C0_b$+	0[1]	1,437	1,325	0,112	0,0319	0,0010	6					✓ 8%
mean C1+[1]	77,59	1,453	1,370	0,083	0,012	0,0001333	3					✓ 6%
mean C2+[1]	25,86	1,447	1,320	0,127	0,012	0,0001333	3					✓ 9%
mean C3+[1]	8,62	1,443	1,307	0,137	0,015	0,0002333	3					✓ 9%
mean C4+[1]	2,87	1,443	1,313	0,130	0,017	0,0003	3					✓ 9%
mean C5+[1]	0,96	1,440	1,330	0,110	0,010	0,0001	3					✓ 8%
mean C6+[1]	0,32	1,450	1,320	0,130	0,017	0,0003	3					✓ 9%
mean C7+[1]	#NV											

Replicate2 1-3

Replicate 1-3 (+Digitonin)

[1]) including Digitonin

phenyl ether: laboratory 5. test run 2 (Lab_5_2)

AS sampling date	04-Sep-12
dry weight	3,6 g/L
test date	05-Sep-12
test substance	phenyl ether

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

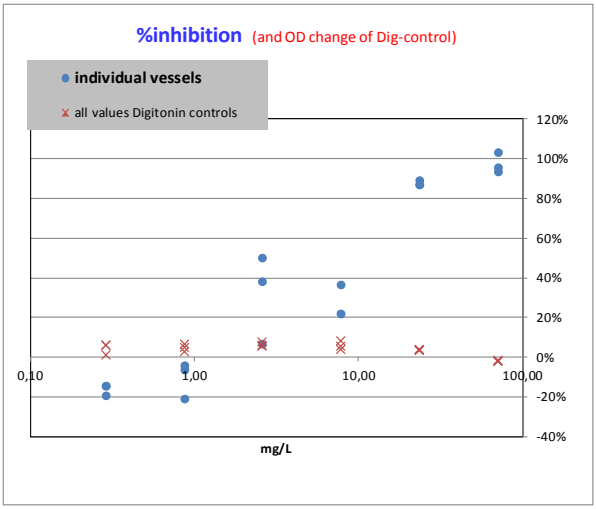
Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,47	0,93	0,54
$C0_b$	control	1,47	0,89	0,58
C1 in test	70,22	1,49	1,53	-0,04
C2 in test	23,41	1,44	1,34	0,1
C3 in test	7,80	1,44	1,03	0,41
C4 in test	2,60	1,45	0,97	0,48
C5 in test	0,87	1,42	0,91	0,51
C6 in test	0,290	1,42	0,88	0,54
C7 in test				

Replicate 2

$C0_a$	control	1,45	0,91	0,54
$C0_b$	control	1,41	0,82	0,59
C1 in test	70,22	1,5	1,51	-0,01
C2 in test	23,41	1,43	1,32	0,11
C3 in test	7,80	1,37	1,02	0,35
C4 in test	2,60	1,38	1,08	0,3
C5 in test	0,87	1,37	0,87	0,5
C6 in test	0,29	1,41	0,87	0,54
C7 in test				

Replicate 3

$C0_a$	control	1,42	1,02	0,4
$C0_b$	control	1,36	0,92	0,44
C1 in test	70,22	1,48	1,48	0
C2 in test	23,41	1,45	1,34	0,11
C3 in test	7,80	1,43	1,02	0,41
C4 in test	2,60	1,44	1,09	0,35
C5 in test	0,87	1,43	0,86	0,57
C6 in test	0,29	1,39	0,83	0,56
C7 in test				



Replicate 1 (+Digitonin)

Code	control	0h-OD440	25h-OD440	ΔOD
$C0_a$+	control1	1,48	1,38	0,1
$C0_b$+	control1	1,49	1,40	0,09
C1+ in test[1]	70,22	1,52	1,55	-0,03
C2+ in test[1]	23,41	1,46	1,41	0,05
C3+ in test[1]	7,8	1,49	1,43	0,06
C4+ in test[1]	2,60	1,47	1,38	0,09
C5+ in test[1]	0,87	1,49	1,42	0,07
C6+ in test[1]	0,29	1,44	1,42	0,02
C7+ in test[1]				

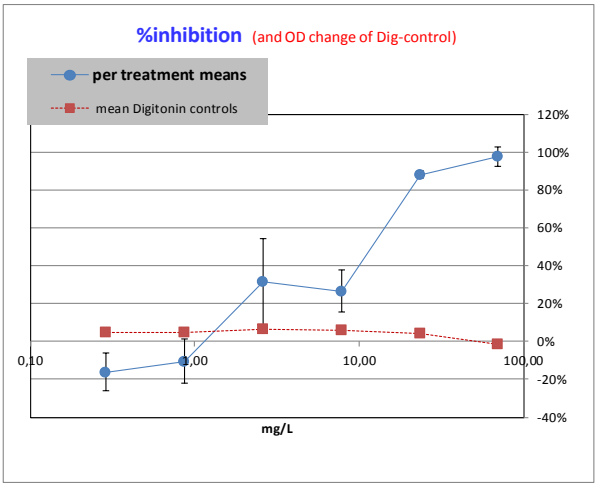
Replicate 2 (+ Digitonin)

$C0_a$+	control1	1,47	1,36	0,11
$C0_b$+	control1	1,48	1,34	0,14
C1+ in test[1]	70,22	1,49	1,52	-0,03
C2+ in test[1]	23,41	1,48	1,42	0,06
C3+ in test[1]	7,8	1,49	1,37	0,12
C4+ in test[1]	2,6	1,47	1,36	0,11
C5+ in test[1]	0,87	1,44	1,4	0,04
C6+ in test[1]	0,29	1,46	1,37	0,09
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control1	1,46	1,36	0,1
$C0_b$+	control1	1,45	1,38	0,07
C1+ in test[1]	70,22	1,48	1,50	-0,02
C2+ in test[1]	23,41	1,46	1,40	0,06
C3+ in test[1]	7,8	1,46	1,38	0,08
C4+ in test[1]	2,60	1,47	1,39	0,08
C5+ in test[1]	0,87	1,46	1,36	0,1
C6+ in test[1]	0,29	1,47	1,38	0,09
C7+ in test[1]				

[1]) including Digitonin



phenyl ether: Lab_5_2 (continued)

	data analysis / evaluation												
	Code	mg/L	$mean_{2h\text{-}OD}$	$mean_{22h\text{-}OD}$	mean ΔOD	StDev	variance	n	mean ΔOD_{corr}	StDev	% inhibition	CV	%OD-decrease
Replicate2 1-3	mean C0	0	1,430	0,915	0,515	0,077	0,006	6	**0,413**	**0,081**	**0%**	20%	✓ **29%**
	mean C1	70,22	1,490	1,507	-0,017	0,021	0,0004	3	**0,010**	**0,022**	**98%**	5%	1%
	mean C2	23,41	1,440	1,333	0,107	0,006	0,0000	3	**0,050**	**0,008**	**88%**	2%	3%
	mean C3	7,80	1,413	1,023	0,390	0,035	0,0012	3	**0,303**	**0,046**	**27%**	11%	21%
	mean C4	2,60	1,423	1,047	0,377	0,093	0,0086	3	**0,283**	**0,094**	**31%**	23%	20%
	mean C5	0,87	1,407	0,880	0,527	0,038	0,0014	3	**0,457**	**0,048**	**-10%**	12%	32%
	mean C6	0,290	1,407	0,860	0,547	0,012	0,0001	3	0,480	0,042	-16%	10%	34%
	mean C7	#NV										**0%**	
Replicate 1-3 (+Digitonin)	mean $C0_b$+	0[1]	1,472	1,370	0,102	0,0232	0,0005	6					✓ 7%
	mean C1+[1]	70,22	1,497	1,523	-0,027	0,006	3,333E-05	3					✓ -2%
	mean C2+[1]	23,41	1,467	1,410	0,057	0,006	3,333E-05	3					✓ 4%
	mean C3+[1]	7,80	1,480	1,393	0,087	0,031	0,0009333	3					✓ 6%
	mean C4+[1]	2,60	1,470	1,377	0,093	0,015	0,0002333	3					✓ 6%
	mean C5+[1]	0,87	1,463	1,393	0,070	0,030	0,0009	3					✓ 5%
	mean C6+[1]	0,29	1,457	1,390	0,067	0,040	0,001633333	3					✓ 5%
	mean C7+[1]	#NV											

[1]) including Digitonin

8 APPENDIX II – Instructions to participants

9.I Preparation of standard dilution medium

I. preparation of *chemical stock solutions* for moderately hard EPA synthetic water*, pH-buffered ($EPA_{mod/buffer}$)

1) prepare 100 mL of a 40-fold concentrated stock solution for each reagent 1-4

reagent	final conc. in test mg/L	conc. factor 40x	reagent per 100 mL	
(1) $NaHCO_3$	96	40	384 mg	ad 100 mL H_2O
(2) $CaSO_4 \cdot 2H_2O$	60	40	240 mg	ad 100 mL H_2O
(3) $MgSO_4$	60	40	240 mg	ad 100 mL H_2O
(4) KCl	4	40	16 mg	ad 100 mL H_2O
	mM			
(5) Hepes, pH 7,5	10	40	9,532 g	adjust pH! → ad 100 mL H2O

2) adjust the pH of the HEPES buffer-solution to pH 7.5 (NaOH)

a) dissolve the HEPES reagent in ca. 90 mL of H_2O

b) adjust the pH to 7.5 with a concentrated NaOH solution (ca. 2.8 mL of a 5 M (200g/L) NaOH solution)

c) fill up to 100 mL with deion. (or distilled) water

3) store the $EPA_{mod/buffer}$ stocks at -20°C

(for practical reasons it is recommended to aliquote the stocks)

II. preparation of the standard moderately hard EPA synthetic water, pH-buffered ($EPA_{mod/buffer}$)

→ Fill a 200 milliliter volumetric flask with approximately 160 ml deionized (or distilled) water and
→ add 5 mL of each concentrated stock solution, in the sequence 1 to 5.
→ Add deionized (or distilled) water up to the 200 ml mark and shake to homogenize the medium.
store the (diluted) $EPA_{mod/buffer}$ medium at 4-6°C for no longer than 2 weeks.

***) EPA synthetic water**

(EPA 821/R-02-013, Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed.October 2002. (http://www.epa.gov/waterscience/WET/disk3)

TABLE 3. PREPARATION OF SYNTHETIC FRESHWATER USING REAGENT GRADE CHEMICALS[1]

Water Type	Reagent Added (mg/L)[2]				Approximate Final Water Quality		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH[3]	Hardness[4]	Alkalinity[4]
Very soft	12	7,5	7,5	0,5	6,4-6,8	10-13	10-13
Soft	48	30	30	2	7,2-7,6	40-48	30-35
Moderately Hard	96	60	60	4	7,4-7,8	80-100	57-64
Hard	192	120	120	8	7,6-8,0	160-180	110-120
Very hard	384	240	240	16	8,0-8,4	280-320	225-245

[1]Taken in part from Marking and Dawson (1973).
[2]Add reagent grade chemicals to deionized water.
[3]Approximate equilibrium pH after 24 h of aeration.
[4]Expressed as mg $CaCO_3$/L.

9.2 test chemicals: description and preparation

No	substance	synonyms	source	CAS-No	MW	phys. state (RT)	density (20°C)	logPow	solvent	tox. range (nominal conc.)	test range (nominal conc.)
1	1-octylamine	Caprylamin, Octanamine	Fluka Order-No. 74988	111-86-4	129,25	Colourless liquid	0,782 g/cm3	0,76	EPA-water (mod/b)[1]	1-100 mg/L	C1=200 mg/L; factor 1:3
2	3,5-dichlorophenol	3,5-DCP	Fluka Order-No. 31595	591-35-5	163,1	crystalline Powder,	-	3.62-3.68	EPA-water (mod/b)[1]	0,1-10 mg/L	C1=20 mg/L; factor 1:3
3	Dimethyl sulfoxide	DMSO	Sigma Order-No. D-5879	67-68-5	78,13	Colourless liquid	1.1004 g cm^{-3}	-1,35	EPA-water (mod/b)[1]	2-50 g/L	C1=100 g/L; factor 1:2,5
4	phenyl ether	Diphenylether, Diphenyl Oxide	Fluka Order-No. 67334	101-84-8	170,21	crystals or liquid depend. on temp.	1.072 g/mL	4,21	EPA-water (mod/b)[1]	1-100 mg/L	C1=100 mg/L; factor 1:3
5	hexachlorophene	HCP	Fluka Order-No. 45526	70-30-4	406,9	crystalline Powder	-	7.54 (exp.) 6.92 (est.)	DMSO[2]	0,1-10 mg/L	C1= 15 mg/L; factor 1:3
	digitonin	Digitin	Sigma Order-No. D5628	11024-24-1	1229	Powder	-		H_2O	1-100 mg/L	200 mg/L

[1]) $EPA_{mod/b}$: EPA-water, moderately hard, pH-buffered with 10 mM HEPES, pH 7,5, NaOH
[2]) DMSO must not exceed 0.2% in test
[3]) Stock solutions of Digitonin in water have to be heated (95 - 100°C) before use, until a clear solution is obtained. After cooling this solution remains clear for hours.

No	substance	preparation	required quantity	
1	1-octylamine	add 20 µL of substance to 78,2 mL of $EPA_{mod/b}$ (= 15,64 mg/78,2 mL = **200 mg/L** = highest test concentration, control dissolving).	ca. 4-6 mL per test	Solution is readily mixed
2	3,5-dichlorophenol	1) add 20 mg of substance 3,5-D to 200 mL of $EPA_{mod/b}$ (= 100 mg/L) 2) dilute 1:5: add 5 mL of this 3,5-DCP stock to 20 mL of $EPA_{mod/b}$ **= 20 mg/L** = highest test concentration.	ca. 4-6 mL per test	Solution is readily mixed
3	Dimethyl sulfoxide	add 2,727 mL (3 g) of DMSO to 27,273 mL of $EPA_{mod/b}$ **= 100 g/L** = highest test concentration.	ca. 4-6 mL per test	**Please prepare**
4	phenyl ether	add 18,66 µL (20 mg) of the substance to 200 mL of $EPA_{mod/b}$ **= 100 mg/L** = highest test concentration; very slightly soluble: make sure that the chemical (oily fluid) is transferred to the dilution medium. To disperse/dissolve **shake vigorously - especially before each use**! Be aware of the volatility of the substance, i.e. keep the vial containing the test chemical closed as far as possible.	ca. 4-6 mL per test	**Please prepare**
5	hexachlorophen	1) add 20 mg of substance HCP to 2,67 mL of DMSO (= 7,5 g/L DMSO). 2) dilute 1:500: add 60 µL of this DMSO stock to 29,94 mL of $EPA_{mod/b}$ **= 15 mg/L** = highest test concentration; make sure that the substance is dissolved, otherwise try to stepwise dilute the highest test concentration until dissolved).	ca. 4-6 mL per test	Solution is readily mixed
	digitonin (defaunation)	add 100 mg digitonin to 10 mL H_2O (= 10 g/L = 50-fold concentrated solution), heat the solution at 95°C for 5 minutes and vortex slowly to dissolve the precipitate. Cool to room temperature prior to use.	ca. 0,3 mL per test (40 µL per tube)	Solution is readily mixed

9.3 EXCEL spreadsheet I – test protocol form

...please fill in the yellow marked areas!

Protozoa Activated Sludge Test

AS sampling date	7-Aug-2012	
AS dry weight	4,4	g/L
AS volume in 2 mL-assay	0,455	mL [Vol_{AS}]
diluent ($EPA_{mod/buffered}$)	1,505	mL [Vol_{dil}]
volume of bacterial food	40	µL (E.coli-suspension)

test substance: 3,5-Dichlorophenol
highest prepared concentration C1: 20 mg/L

dilution factor: 1: 3 (preferably beween 2,0 and 3,2)
(covering a 81-fold concentration range)
from 20 to 0,247 mg/L

prepare 42 vessels per test chemical (14 vessels per replicate, 3 replicates in total):

per replicate: 2 controls and 5 test dilutions without and
2 controls and 5 test dilutions with Digitonin (defaunated)

one replicate!		
C0	C0+	Controls without and with Digitonin (+)
C0	C0+	Controls without and with Digitonin (+)
C1	C1+	highest prepared concentration without and with Digitonin (+)
C2	C2+	.
C3	C3+	.
C4	C4+	.
C5	C5+	lowest prepared concentration without and with Digitonin (+)

1. fill the controls (vessels C0) and the test vessels C2-C5 with 1,505 mL diluent [Vol_{dil}]

2 prepare dilution series:

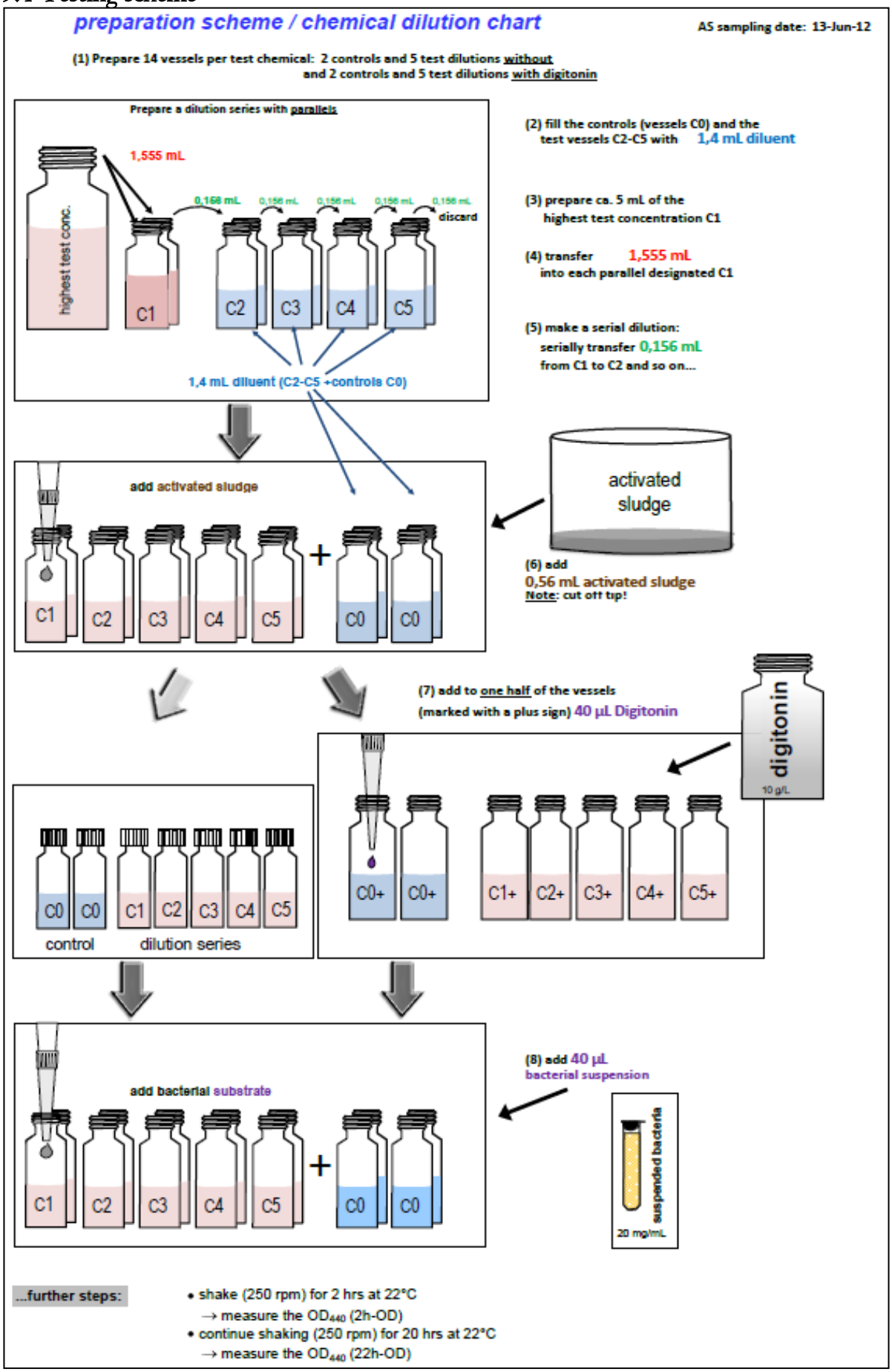
add	2,258	mL of highest test solution C1 to vessel 1 [Vol_{C1}]
transfer	0,753	mL of test solution C1 from vessel 1 to vessel 2 [Vol_{trans}]
transfer	0,753	mL of test solution C2 from vessel 2 to vessel 3 [Vol_{trans}]
transfer	0,753	mL of test solution C3 from vessel 3 to vessel 4 [Vol_{trans}]
transfer	0,753	mL of test solution C4 from vessel 4 to vessel 5 [Vol_{trans}]
discard	0,753	mL of test solution C5 [Vol_{trans}]
add	0,455	mL of activated sludge[1] to each vessel [Vol_{AS}]
add	40	µL of Digitonin solution[2] to all + labelled vessels
add	40	µL of E.coli-suspension[3] to each vessel

1) AS-inoculum: 1g/L dw
2) digitonin-solution: 200 mg/L
3) E.coli-suspension: 0,4 mg/mL)

...further steps:

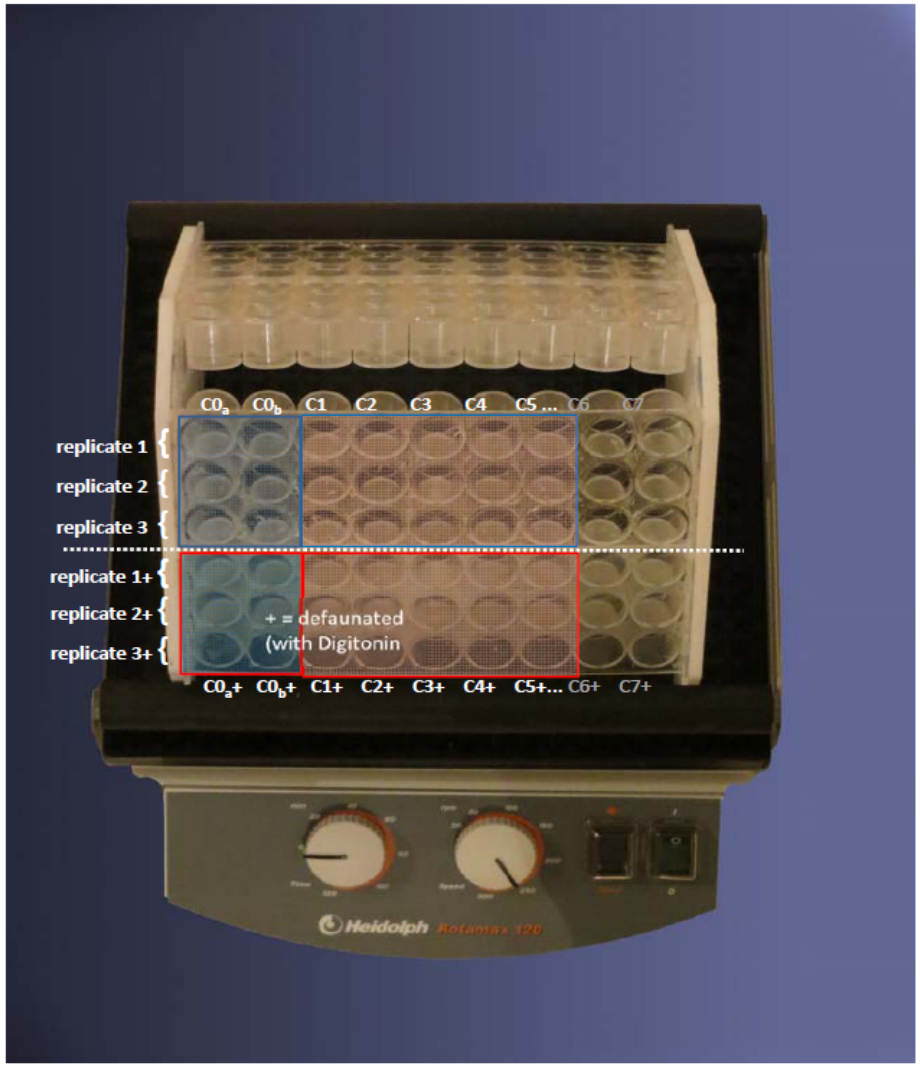
- shake (250 rpm) for 2 hrs at 22°C
  → measure the OD_{440} (2h-OD)
- shake (250 rpm) for 20 hrs at 22°C
  → measure the OD_{440} (22h-OD)

9.4 Testing scheme



9.5 Proposed placement of test vials in the holding tray

maximal 2 test chemicals with
maximal 7 test concentrations



- test tube rack -
(possible placement of test tubes)

replicate 1	$C0_a$	$C0_b$	C1	C2	C3	C4	C5	C6	C7
replicate 2	$C0_a$	$C0_b$	C1	C2	C3	C4	C5	C6	C7
replicate 3	$C0_a$	$C0_b$	C1	C2	C3	C4	C5	C6	C7
replicate 1+	$C0_a$+	$C0_b$+	C1+	C2+	C3+	C4+	C5+	C6+	C7+
replicate 2+	$C0_a$+	$C0_b$+	C2+	C2+	C3+	C4+	C5+	C6+	C7+
replicate 3+	$C0_a$+	$C0_b$+	C3+	C2+	C3+	C4+	C5+	C6+	C7+

21 test tubes: 6 controls + 5 concentration steps in triplicate
21 test tubes: 6 defaunated controls + 5 defaunated concentration steps in triplicate

9.6 EXCEL spreadsheet 2 – exemplary documentation form

...please fill in the yellow marked areas!

AS sampling date 07-Aug-12
dry weight 4,4 g/L

test date 09-Aug-12
test substance 3,5-Dichlorophenol

! For a statistically acceptable evaluation, each test
! concentration has to be assayed in three replicates!
! Accordingly, the controls are assayed in six replicates.

...please fill in the yellow marked areas!

	Code	defaunated[1]	Code
control	$C0_a$	control	$C0_a$+
control	$C0_b$	control	$C0_b$+
highest conc.	C1	highest conc.	C1+
	C2		C2+
	C3		C3+
	C4		C4+
lowest conc.	C5	lowest conc.	C5+

[1]) including Digitonin

Replicate 1

Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$	control	1,43	0,94	0,49
$C0_b$	control	1,46	0,91	0,55
C1 in test	15,05	1,48	1,53	-0,05
C2 in test	5,02	1,52	1,37	0,15
C3 in test	1,67	1,46	1,2	0,26
C4 in test	0,56	1,44	1,04	0,4
C5 in test	0,19	1,47	1,03	0,44
C6 in test	0,060	1,43	1,01	0,42
C7 in test				

Replicate 2

$C0_a$	control	1,44	0,95	0,49
$C0_b$	control	1,44	0,98	0,46
C1 in test	15,05	1,52	1,57	-0,05
C2 in test	5,02	1,48	1,33	0,15
C3 in test	1,67	1,37	1,18	0,19
C4 in test	0,56	1,41	0,97	0,44
C5 in test	0,19	1,45	0,97	0,48
C6 in test	0,06	1,41	0,95	0,46
C7 in test				

Replicate 3

$C0_a$	control	1,43	0,99	0,44
$C0_b$	control	1,43	0,98	0,45
C1 in test	15,05	1,52	1,54	-0,02
C2 in test	5,02	1,45	1,31	0,14
C3 in test	1,67	1,47	0,98	0,49
C4 in test	0,56	1,47		
C5 in test	0,19	1,48	1,01	0,47
C6 in test	0,06	1,43	0,93	0,5
C7 in test				

Replicate 1 (+Digitonin)

Code	mg/L	0h-OD_{440}	25h-OD_{440}	ΔOD
$C0_a$+	control[1]	1,47	1,32	0,15
$C0_b$+	control[1]	1,48	1,31	0,17
C1+ in test[1]	15,05	1,58	1,64	-0,06
C2+ in test[1]	5,02	1,51	1,44	0,07
C3+ in test[1]	1,67	1,47	1,32	0,15
C4+ in test[1]	0,56	1,45	1,3	0,15
C5+ in test[1]	0,19	1,45	1,31	0,14
C6+ in test[1]	0,06	1,47	1,32	0,15
C7+ in test[1]				

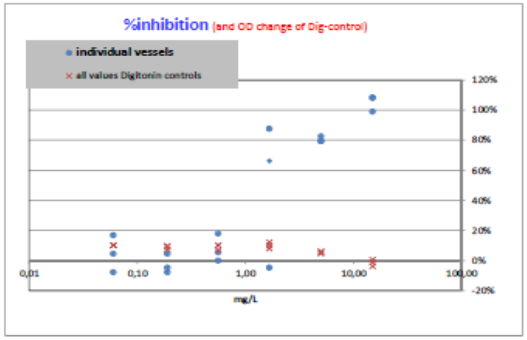
Replicate 2 (+ Digitonin)

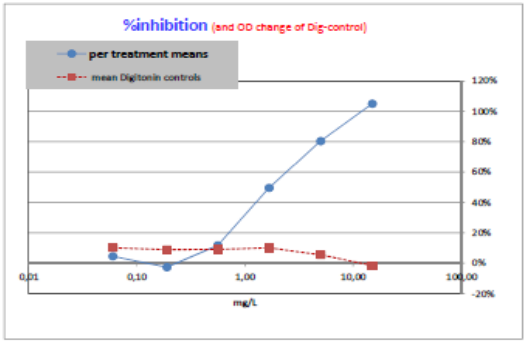
$C0_a$+	control[1]	1,46	1,31	0,15
$C0_b$+	control[1]	1,46	1,29	0,17
C1+ in test[1]	15,05	1,58	1,6	-0,02
C2+ in test[1]	5,02	1,5	1,41	0,09
C3+ in test[1]	1,67	1,49	1,37	0,12
C4+ in test[1]	0,56	1,47	1,32	0,15
C5+ in test[1]	0,19	1,43	1,32	0,11
C6+ in test[1]	0,06	1,47	1,32	0,15
C7+ in test[1]				

Replicate 3 (+Digitonin)

$C0_a$+	control[1]	1,47	1,32	0,15
$C0_b$+	control[1]	1,48	1,34	0,14
C1+ in test[1]	15,05	1,59	1,58	0,01
C2+ in test[1]	5,02	1,5	1,41	0,09
C3+ in test[1]	1,67	1,48	1,3	0,18
C4+ in test[1]	0,56	1,47	1,37	0,1
C5+ in test[1]	0,19	1,46	1,32	0,14
C6+ in test[1]	0,06	1,47	1,32	0,15
C7+ in test[1]				

[1]) including Digitonin





				+ Digitonin (defaunated)		
	replicate1	replicate2	replicate3	replicate1	replicate2	replicate3
x (mg/L)	y (% Effekt)	y (% Effekt)	y (% Effekt)	y (% Effekt)	y (% Effekt)	y (% Effekt)
15,05	108%	108%	99%	-4%	-1%	1%
5,02	79%	79%	83%	5%	6%	6%
1,67	66%	88%	-5%	10%	8%	12%
0,56	18%	6%	#WERT!	10%	10%	7%
0,19	5%	-8%	-5%	10%	8%	10%
0,06	17%	5%	-8%	10%	10%	10%
	#WERT!	#WERT!	#WERT!	#WERT!	#WERT!	#WERT!

	mean values	
x (mg/L)	y (% Effekt)	y (% Effekt)
15,05	105%	-1%
5,02	81%	6%
1,67	50%	10%
0,56	12%	9%
0,19	-3%	9%
0,06	5%	10%

9 APPENDIX III - SOP

SOP (Standard Operating Procedure)

Testing chemical compounds

Glass TEST TUBES containing standard dilution water[16] with the desired concentration of the test substance are inoculated with an aliquot of activated sludge to give the desired activated sludge concentration of 1 g (dry weight) per L in a final assay volume of 2 mL.

If the approximate toxicity of the chemical compound to the phagocytotic activity of activated sludge is known, a DEFINITIVE TEST can be performed immediately. However, if no data is available on the toxicity of the chemical, two consecutive assays must be performed:

a) a range finding test to determine the 0-100% tolerance range of the activated sludge to the toxicant.
b) a definitive test to determine the 50% inhibition threshold with more precision.

Equipment / Material

- glass test tubes (45 mm long and 15 mm in external diameter. total volume of 4 mL) with oxygen permeable caps.
- Analytical balance
- standard dilution water16. possibly dimethyl sulfoxide (DMSO)
- variable pipettes: 2 mL. 1 mL. 100 µL. 10 µL (preferably positive displacement µL-pipettes). pipette tips
- racks enabling a slanting position of test tubes with an angle of inclination between of 20-40° to horizontal.
- digital photometer (filter of λ=440 nm) suitable for above mentione

Preparation of Chemical Test Solutions

In case of readily and moderately soluble substances prepare a solution with the highest soluble concentration of the test chemical in the standard dilution water (prepare freshly before each test run). To avoid inaccuracies of weighing (solids) or pipetting low volumes (solutions) do not fall below 20 mg and 40 µL. respectively.

[16] buffered synthetic water ($EPA_{moderately\ hard}$, pH-buffered with 10 mM Hepes, pH7.5)

Poorly soluble compounds may be dissolved in organic solvents, preferably dimethyl sulfoxide (DMSO). The concentration of the vehicle must not exceed 0.1 mL/L in the test, i.e. the stock solution in the organic solvent must be 10,000-fold diluted in standard dilution water16 to gain the highest chemical concentration for testing purposes.

Preparation of Samples for range finding test

Prepare a dilution series by serial dilution with standard dilution water[16], starting with the highest soluble concentration (or the highest soluble concentration containing no more than 0.2% vehicle).

1) Prepare the highest concentration to be tested in a small glass vessel (10-100 mL).
2) Take 14 glass test tubes and label them C0 (duplicates) to C5 and C0+ (duplicates) to C5+.
3) C0 represents the controls and C1 to C5 the chemical dilution series. The plus sign denotes the addition of digitonin.
4) Determine the volume of the activated sludge (vol_{AS}) to be added to the test vials to obtain a concentration of 1g/L for the 2 mL-assays[17].
5) Specify the diluent volume (vol_{dil}) on the basis of vol_{AS} and the substrate volume of 40 µL (vol_{dil} = 2 mL minus vol_{AS} minus 40 µL) and prepare the 10-fold dilution series directly in the test vials[18].

Table: Exemplary 10-fold dilution series of the chemical compound

Vessels	chemical concentration mg/L
C1	100
C2	10
C3	1
C4	0.1
C5	0.01

…proceed to section 'Inoculation of the test vials'.

[17] e.g. the concentration of native activated sludge amounts to 3.57 g/L dry weight. To obtain a sludge concentration of 1 g/L the native sludge has to be diluted by a factor of 1:3.57, i.e. in the 2 mL-assays 0.56 mL (2 mL*1/3.57) native activated sludge have to be added (→ preparation sheet & preparation scheme).

[18] using the same example the diluent volume accounts for 1.40 mL (2 mL assay volume - 0.56 mL vol_{AS} – 40 µL substrate volume = 1.40 mL). To prepare e.g. a 1:10 dilution series fill both C1 test tubes with 1.556 mL (=1.40 mL plus 0.156 mL: nine parts + one part corresponds to a dilution of 1:10) of the highest test concentration (stock) and add to all remaining test tubes (controls and chemical dilutions) 1.40 mL standard dilution water. Afterwards, transfer 0.156 mL (1 part) of the highest test concentration (stock) to the 1.40 mL (nine parts) standard dilution water in C2 and mix (dilution1). Then, use dilution 1 to prepare dilution 2 etc. (→ preparation sheet & preparation scheme).

Preparation of samples for definitive test

The dilution series to be prepared should span the range of the lowest concentration producing 80-100% effect and the highest concentration producing less than 10% effect in the range-finding test.

This range can span one order of magnitude (case A) or two orders of magnitude (case B).

The new concentration range to be tested will again be called C1-C5.

Note: each definitive test contains three replicates.

A. C1-C5 spans one order of magnitude

Prepare a dilution series by serial 1:2 dilution with diluent16 starting with the lowest chemical concentration that produced 80-100% inhibition in the range finding test.

1) Prepare the highest concentration to be tested in a small glass vessel (10-100 mL).
2) Take 42 glass test tubes (=14 tubes per replicate x 3 replicates) and label each replicate with C0 (duplicates) to C5 and C0+ (duplicates) to C5+. C0 represents the controls and C1 to C5 the chemical dilution series. The plus sign denotes the addition of digitonin.
3) Determine the volume of the activated sludge (vol_{AS}) to be added to the test vials to obtain a concentration of 1g/L for the 2 mL-assays[19].
4) Specify the diluent volume (vol_{dil}) on the basis of vol_{AS} and the substrate volume of 40 µL (vol_{dil} = 2 mL minus vol_{AS} minus 40 µL) and prepare the 1:2 dilution series directly in the test vials[20].

…proceed to section 'Inoculation of the test vials'.

[19] e.g. the concentration of native activated sludge amounts to 3.57 g/L dry weight. To obtain a sludge concentration of 1 g/L the native sludge has to be diluted by a factor of 1:3.57, i.e. in the 2 mL-assays 0.56 mL (2 mL*1/3.57) native activated sludge have to be added.

[20] using the same example the diluent volume accounts for 1.40 mL (2 mL assay volume - 0.56 mL vol_{AS} – 40 µL substrate volume = 1.40 mL). To prepare e.g. a 1:2 dilution series fill both C1 test tubes with 2.8 mL (=1.40 mL plus 1.40 mL: one parts + one part corresponds to a dilution of 1:2) of the highest test concentration (stock) and add to all remaining test tubes (controls and chemical dilutions) 1.40 mL standard dilution water. Afterwards, transfer 1.40 mL (1 part) of the highest test concentration (stock) to the 1.40 mL (1 part) standard dilution water in C2 and mix (dilution1). Then, use dilution 1 to prepare dilution 2 etc.

B. C1-C5 spans two orders of magnitude

Prepare a dilution series by serial 1:3 dilution with standard dilution water16 starting with the lowest chemical concentration that produced 80-100% inhibition in the range finding test.

1) Prepare the highest concentration to be tested in a small glass vessel (10-100 mL).
2) Take 42 glass test tubes (=14 tubes per replicate x 3 replicates) and label each replicate with C0 (duplicates) to C5 and C0+ (duplicates) to C5+. C0 represents the controls and C1 to C5 the chemical dilution series. The plus sign denotes the addition of digitonin.
3) Determine the volume of the activated sludge (vol_{AS}) to be added to the test vials to obtain a concentration of 1g/L for the 2 mL-assays[21].
4) Specify the diluent volume (vol_{dil}) on the basis of vol_{AS} and the substrate volume of 40 µL (vol_{dil} = 2 mL minus vol_{AS} minus 40 µL) and prepare the 1:3 dilution series directly in the test vials[22].

…proceed to section 'Inoculation of the test vials'.

Inoculation of the test vials

1) Take the activated sludge container[23] and shake it gently to homogenize the sludge suspension.
2) Transfer the respective volume of the activated sludge (vol_{AS}. see above under '*PREPARATION OF SAMPLES FOR RANGE FINDING TEST*') to the test vials by means of an automatic pipette. To avoid floc-destroying shear forces do not forget to cut off the pipette tip about 5 mm from the end enlarging the tip opening to ca. 3 mm.

…proceed to section 'ADDITION OF SUBSTRATE'.

Addition of digitonin and substrate

1) bring Digitonin into solution and add 40 µL this 10 g/L solution to one half of the test vials (C0+ controls and chemical dilution series C1+ to C5+),

---

[21] e.g. the concentration of native activated sludge amounts to 3.57 g/L dry weight. To obtain a sludge concentration of 1 g/L the native sludge has to be diluted by a factor of 1:3.57, i.e. in the 2 mL-assays 0.56 mL (2 mL*1/3.57) native activated sludge have to be added.

[22] using the same example the diluent volume accounts for 1.40 mL (2 mL assay volume - 0.56 mL vol_{AS} – 40 µL substrate volume = 1.40 mL). To prepare e.g. a 1:3 dilution series fill both C1 test tubes with 2.1 mL (=1.40 mL plus 0.7 mL: two parts + one part corresponds to a dilution of 1:3) of the highest test concentration (stock) and add to all remaining test tubes (controls and chemical dilutions) 1.40 mL standard dilution water. Afterwards, transfer 0.70 mL (1 part) of the highest test concentration (stock) to the 1.40 mL (two parts) standard dilution water in C2 and mix (dilution1). Then, use dilution 1 to prepare dilution 2 etc.

[23] for testing purposes activated sludge probes (sampling: see → video1) can be stored in the refrigerator (4-6°C) and should be used within one week.

2) reconstitute the freeze-dried bacterial substrate with H_2O to obtain a 50-fold concentrated bacterial solution of 20 mg(dw)/mL and
3) pipette 40 µL of the substrate solution to each test vial,
4) close all test vials with screw caps,
5) put all test vials in the holding tray and place on a shaker (250 rpm) at (22 ± 1°C). Be aware of the inclination of the vials in the rack (20-40° to horizontal),
6) equilibrate the probes for 2 hours while shaking (250 rpm).

…proceed to section 'Optical density measurements'.

Optical density measurements

1) remove the test vials from the shaker. Mix each vial by vortexing for 3 seconds at highest speed. Subsequently. let the vials stand for 30 min to settle solids.
2) After 30 min of sedimentation the optical density of the supernatant is measured in the test vials:
   a) Zero-calibrate the photometer at 440 nm with a test vial (blank) containing exclusively 2 mL of standard dilution water16.
   b) Measure the optical density of the probes at 440.

Note: The photometer cuvette holder must be built so that the light beam passes through the supernatant above the sedimented sludge.

Be careful placing the test vials into the cuvette slot. The activated sludge must remain at the bottom of the test vials to not disturb the optical readings.

3) Record the data of the first (2 hours-) OD measurements (2h-OD).
4) Put all test vials in the holding tray again and continue shaking (250 rpm. 22 ± 1°C. 20-40° inclination of the vials to horizontal).
5) After 22 hours incubation determine once again the optical density of each test tube:
   a) remove the test vials from the shaker. Mix each vial by vortexing for 3 seconds at highest speed and let the vials stand for 30 min to settle solids
   b) Following settling of solids zero-calibrate the photometer and measure the optical density of the probes
6) Record the data of the second (22hours-) OD measurement (22h-OD).

Validity of the test

For a test to be valid the following conditions should be fulfilled:

- controls: the averaged decrease of the optical density of (untreated) controls due to phagocytotic activities (% mean ΔOD_{corr}(controls)) should at least exceed 25% within the testing time (if the 25% criterion is not fulfilled, a warning appears in the → EXCEL form).
- protozoa-free controls: the (unspecific) decrease of the optical density of defaunated (digitonin-) controls should not exceed 25% within the testing time (-> EXCEL form).
- chemically treated protozoa-free controls: in some cases test chemicals may cause an increase of the optical density of Digitonin-controls within the testing period especially at high concentrations. Concentrations at which this occurs should not be included in the effect calculation, i.e. in the evaluation of concentration-response curves if the increase amounts to more than 5%.

Troubleshooting guide

- low phagocytotic activity i.e. the optical density of (untreated) controls decreases less than 25% within the testing time (% mean ΔOD_{corr}(controls) < 25%):

  → low ambient temperatures can – with some time delay - dramatically lower the temperatures of the aeration tanks and correspondingly the phagocytotic activity of activated sludge (in Central Europe particularly in late winter and early spring). However, a phagocytotic activity below 25% is considered exceptional and – according to the experience of the validation management - paralleled by a low metabolic activity of the sludge as a whole. In case of an extended period of low activity it is advisable to take the activated sludge from another wastewater treatment plant.

- high OD-decrease of defaunated (Digitonin-) controls:

  → Attachment of microorganisms on surfaces is a common and well-known behavior in both natural and engineered aquatic environments. In the test this effect can be observed as a non-phagocytosis mediated decrease in the optical density (turbidity). If the decrease of defaunated controls (%mean ΔOD_{def}(controls)) exceeds 25% within the testing period the following factors influencing this 'unspecific' decrease should be examined: integrity of bacterial food (is the bacterial powder still dry?); shaking speed high enough (250 rpm) during incubation? equilibrated (shaking at 250 rpm and at 22°C) for 2 hours before the first OD-measurement? samples vigorously vortexed and subsequently settled for 30 min just before the OD-measurement? If none of these factors apply a new batch of lyophilized *E.coli*-powder should be used for further testing.

- increase of OD at high chemical concentrations:

  → at high test chemical concentrations an increase of the turbidity may occur. If the increase of defaunated and chemically treated probes [i.e. Digitonin-controls containing test chemicals: (%mean ΔOD_{def}(treatment))] exceeds 5% the test result is not valid and has to be excluded from further evaluation (effect calculation and concentration-response estimation).

Floating sludge / floating scum layer

Avoid floating scum when collecting activated sludge. Do not collect the sample in dead corners where scum has built up. Take your sample by complete immersion of your sampling tool below the surface of the water.

If floating occurs during testing gentle tilting the glass vials may be successful to let the surface layer drop to the bottom.

10 APPENDIX IV – Draft Test Guideline

PROPOSAL FOR A PROTOZOAN ACTIVATED SLUDGE INHIBITION TEST

INTRODUCTION

1. This Test Guideline describes a method to assess effects of a substance on the phagocytotic activity (consumption of dispersed bacteria) of activated sludge under defined conditions in the presence of different concentrations of the test substance.
2. The principle of biological sewage-treatment plants is to transform the organic matter of incoming wastewater in microbial biomass, which in turn is separated from the liquid yielding a purified effluent. The aim of this process is to achieve a maximal reduction of the organic load with a minimal bio-sludge production. The phagocytotic activity of activated sludge organisms supports this process. In conventional plants ciliates usually dominate this activity. It is especially their feeding on bacteria which clarifies the waste water, resulting in a higher transparency, i.e. lower organic loads in the output water.
3. The purpose of the test is to provide a means to record effects of substances on consumers in sewage treatment plants, which consist mainly of ciliated protozoa and which – due to their grazing on bacteria - considerably contribute to the functioning of STPs.
4. The test is most easily applied to water soluble substances which, under the test conditions, are likely to remain in the water.
5. This guideline proposal is based on an international ring study commissioned by the German Federal Environment Agency and conducted in 2011-2013.

PRINCIPLE OF THE TEST

6. The activated sludge sample is exposed to the test chemical in small glass test vials with a culture volume of 2 mL. The vials are closed with oxygen permeable caps and shaken for 22 hrs at 22°C. At the start, the sludge samples are fed suspended bacteria. Whereas the turbidity decreases in control samples, in the chemically inhibited samples no elimination of bacteria takes place. To quantify the phagocytotic activity, the decline of the added bacteria with time is followed by two photometric measurements (λ = 440 nm) after 2 hrs (t1) and after 22 hrs (t2) of incubation. Based on the difference between both values the percentage reduction of the activity in relation to an untreated control is calculated. The test is typically used to determine the EC_x (e.g. EC_{50}) of the test substance. To compensate for unspecific reactions of the added bacterial substrate with the sludge flocs (e.g. binding) or effects of chemicals on the turbidity of the sludge sample (e.g. by disaggregation) defaunated (and thus phagocytosis-free) parallel samples are included for controls and for each concentration.

INFORMATION ON THE TEST SUBSTANCE

7. It is necessary to know the water solubility of the substance under the conditions of the test. A reliable analytical method for the quantification of the substance in the test solutions should also be available.

8. Useful information includes structural formula, degree of purity, chemical stability, dissociation constants, n-octanol/water partition coefficient, vapour pressure and biodegradability (see Guideline 301). Solubility and vapour pressure can be used to calculate Henry's constant which will indicate if losses of the test substance may occur.

VALIDITY OF THE TEST

9. For a test to be valid the following conditions must be fulfilled:

- the decrease of the optical density (OD) of controls due to phagocytotic activity must exceed 30% within the testing time between 2 hrs and 22 hrs (cf. para 42) .

- the unspecific decrease of the OD (defaunated controls containing bacterial substrate) must not exceed 25% within the testing time (cf. para 43).

- chemical concentrations at which an average OD-increase of more than 5% in the defaunated parallel samples occurs has to be excluded from the effect calculation (cf. para 44).

REFERENCE SUBSTANCES

10. A reference substance may be tested as a means of detecting unsatisfactory test conditions. In a 2011 international ring study (present report) the EC_{50} of 3,5-DCP was found to be in the range 1.5 mg/L to 5.1 mg/L for the activated sludge phagocytosis inhibition. If a reference substance is used, the results should be given in the test report.

APPLICABILITY OF THE TEST

11. The test method may be applied to water-soluble, poorly soluble and volatile substances. However,. it may not always be possible to obtain EC_{50} values with chemicals of limited solubility and - although the described procedure uses closed test vessels - valid results with volatile chemicals may only be obtained providing that the bulk (> 80%) of the test substance remains in the reaction mixture at the end of the exposure period. Additional analytical support data should be submitted to refine the EC_X concentration when there is any uncertainty regarding the stability of the test substance or its volatility.

DESCRIPTION OF THE METHOD

Apparatus

12. Normal laboratory equipment and especially the following is necessary:

(a) glass vials with oxygen permeable caps,

recommended are:

- clear glass screw top vials, 45 x 15 mm (outer dimensions), total volume of 4 mL.
- open top closures (screw caps open tops) with teflon faced silicone liners.

(b) a rotary shaker (speed adjustable to 250 rpm) with racks enabling a slanting position of test vials with an angle of inclination of 20°-40° to horizontal.

(c) digital photometer (filter of λ=440 nm) suitable for above men

(d) a cabinet or chamber, in which a temperature of 22°C (± 1°C) can be maintained.

Inoculum

13. Activated sludge from the exit of the aeration stage of a well-operated sewage treatment plant treating predominantly domestic sludge is used as the inoculum for the phagocytosis test. Avoid floating scum when collecting activated sludge. Do not collect the sample in dead corners where scum has built up. Take your sample by complete immersion of your sampling tool below the surface of the water. If floating occurs during testing gentle tilting of the glass vials or manual stirring using a glass rod may be successful to let the surface layer drop to the bottom.

14. To perform the test it is necessary to know the dry weight of the sludge. Suspended solids concentrations of 2 g/L to 4 g/L may be considered appropriate. In most cases wastewater facilities continuously perform routine measurements of the sludge concentration. If actual dry weight data are not available, the dry weight has to be determined. From this result, the volume of native activated sludge suspension can be calculated. This must be suspended in the 2 mL assays in order to obtain an activated sludge with a mixed liquor suspended solids value of 0.9 g/litre.

15. The activated sludge should be used on day of collection. If this is not possible, the whole batch of native - undiluted - sludge should be stored in the refrigerator at 4-7°C for one week maximum.

Substrate

16. Bacteria showing a low tendency to aggregate, to flocculate and to adhere to activated sludge flocs may be used as substrate. Bacteria which meet these requirements and have been found to be suitable as food source for phagotrophic sludge inhabitants are shown in APPENDIX V. However, it should be noted that any other bacterial strains can be used if they fulfill the validity criteria.

17. On the basis of the results of the ring test a final concentration of the bacteria to be used for feeding the sludge of 0.36 g/L (dry weight) can be recommended. This concentration ensures an adequate food supply and reliable OD-measurements (c.f. here paragraph 4.2, page 56).

Test medium

18. To dilute the activated sludge and to prepare the chemical dilution series EPA synthetic water is recommended (the detailed composition of the recommended test medium is given in Appendix VI - Test medium).
19. Modification of the test medium may be necessary for certain purposes, e.g. testing at different pH values. Use of modified media must be described in detail and justified.

Phagocytosis-free controls

20. Some part of the bacterial substrate may form aggregates or attach to the sludge flocs thereby changing the turbidity of supernatant suspension. In addition test chemicals – especially at high concentrations - may have impacts on the floc structure of the activated sludge, leading to an increase in turbidity. Controls without phagocytic activity are therefore necessary, reflecting changes in turbidity due to passive, non-specific reactions in the test system. The eukaryotic inhibitor digitonin (Mooney, 1988) has proved to be a useful and specific inhibitor of phagocytosis completely defaunating the activated sludge probe. To obtain phagocytosis-free data parallels with digitonin at a final of 200 mg/L (preparation see APPENDIX VII - Preparation of digitonin solution) should be included in the test for both controls and all chemical concentrations.

Test substance

21. Pre-dilution series of test solutions are freshly prepared at the start of the study by dilution of a stock solution in test medium. In case of sparingly water soluble substances ultrasonic dispersion or other suitable physical means are the preferred methods to dissolve the test chemical. In some cases the use of solvents may be required in order to produce a suitably concentrated stock solution. Examples of solvents that may be used as vehicles to dissolve substances of low water solubility are acetone, ethyl alcohol and dimethyl sulfoxide. Due to its low toxicity dimethyl sulfoxide may be given preference. However, every effort should be made to avoid the use of such materials and if organic solvents are used, they must not significantly inhibit the phagocytotic activity.
22. The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH it is advised that the test be repeated with pH adjustment and the results reported. In that case the pH value of the stock solution should be adjusted to neutral pH (HCl and NaOH may be used for this adjustment if warranted). This pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test substance is caused.

Conditions of exposure

23. Duration:	22 hours under permanent shaking.
Vessels:	glass vials with oxygen permeable screw caps.
Light:	the test should be performed in the dark.
Temperature:	22 ± 1°C.
Oxygen supply:	a sufficient oxygen supply has to be maintained throughout the test period by fast shaking the test vials at 250 rpm. For the recommended assay the culture volume

should not exceed 2 mL in the (4 ml-) test vials which should be closed with oxygen permeable caps and kept at an angle of 40° to the horizontal.

Replicates and controls

24. The test design should include three replicates at each test concentration and twice that number of controls.

25. For each assay, a parallel phagocytosis-free test run with the same number of samples (triplicates at each test concentration and 6 controls) containing digitonin as specific inhibitor[24] (200 mg/L final concentration) should be included.

26. When a solvent is used to solubilize the test substance, additional controls containing the solvent at the same concentration as used in the test cultures should be included in the test design.

27. A separate set of test solutions may be prepared for analytical determinations of test substance concentrations (see paragraphs 32-34).

28. No less than five concentrations are tested simultaneously, preferably arranged in a geometric series. The lowest concentration should have no observed effect on growth. The highest concentration tested should inhibit growth by at least 50% relative to the control and, preferably, stop growth completely. For statistical reasons. However, it is desirable to select the concentrations so that they bracket the 50% effect level.

Measurements

29. The tests run for 22 hours. After vigorous shaking the test vials and settling the sludge for 30 min the optical density of the supernatant is measured spectrophotometrically (440 nm) after 2 hrs (t1) and after 22 hrs (t2) of incubation. Since under the experimental conditions optical density is directly proportional to the bacteria content, absorbance is a rapid means of non-invasively estimating the phagocytotic activity of sludge samples.

Range finding

30. If no data is available on the toxicity of the chemical, a range finding test is carried out to determine the 0-100% tolerance range of the phagocytotic activity to the toxicant. It should include at least 5 dilution steps with a dilution factor of 10 from a starting concentration of 1000 mg/L or the maximum solubility of the substance.

Limit test

31. Under some circumstances, e.g. when a preliminary test indicates that the test substance is non-toxic at concentrations up to 100 mg/L or up to its limit of solubility in the assay (whichever is the lower), a limit test involving a comparison of responses in a control group and one treatment group (100 mg/L or a concen-

[24]The detergent digitonin selectively renders the eukaryotic plasma membrane permeable but not bacterial cells (Mooney, 1988). The addition of digitonin provides on the one hand a control with total inhibition of the phagocytic activity and on the other it allowed the measurement of any passive reaction of the added bacterial suspension, i.e. the nutrient substrate.

tration equal to the limit of solubility) may be undertaken. It is strongly recommended that this be supported by analysis of the exposure concentration. All previously described test conditions and validity criteria apply to a limit test, with the exception that the number of treatment replicates should be doubled. Growth in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test.

Analytical determinations

32. In some cases, it may be necessary to determine the concentration of the test substance in the test vials (e.g. for highly volatile or strongly adsorbing test substances). Analysis at the start and end of the test of a low and high test concentration and a concentration around the expected EC_{50} may be sufficient where it is likely that exposure concentrations will vary less than 20% from nominal values during the test. Analysis of all test concentrations at the start and end of the test is recommended where concentrations are unlikely to remain within 80-120% of nominal (e.g. for volatile or strongly adsorbing test substances). In all cases, determination of test substance concentrations need only be performed on one replicate vessel at each test concentration (or the contents of the vessels pooled by replicate).

33. Test samples prepared specifically for analysis of exposure concentrations should be treated identical to those used for testing, i.e. they should be inoculated with activated sludge, provided with food and incubated under identical conditions. If analysis of the dissolved test substance concentration is required, it may be necessary to separate the solid constituents from the aqueous phase. Separation should preferably be made by centrifugation, sufficient to settle the activated sludge and the suspended bacterial food substrate.

34. If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within $\pm$ 20% of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial. If the deviation from the nominal or measured initial concentration is greater than $\pm$ 20%, analysis of the results should be based on average concentration during exposure.

Other observations

35. Microscopic observation can be performed to verify a normal and healthy appearance of the inoculum activated sludge and to observe any abnormal appearance of the bacterial feeders, especially protozoans.

DATA AND REPORTING

Response variables

36. The purpose of the test is to determine the effects of the test substance on the phagocytosis activity of activated sludge.

Treatment of results:

37. The content of suspended bacterial food in the test vessels is expressed in units of the surrogate parameter optical density (OD_{440}) used for measurement.

38. The measured optical densities in the test cultures and controls are tabulated together with the concentrations of the test substance and the time of measurement.

39. The phagocytotic activity is calculated on the basis of the difference between the initial optical density after 2 hrs of incubation and that after 22 hours of incubation;

i.e.: ΔOD = optical density difference between 2 and 22 hours (controls and treatments without digitonin).

40. To correct for unspecific optical density changes (e.g. due to binding. complexing or lysis of the bacterial food or due to deflocculating effects of test chemicals) defaunated, digitonin-treated parallels without phagocytotic activity are included in the test design. Their shift in optical density values between 2 hrs and 22 hours (ΔOD_{def}) is subtracted from the ΔOD-values;

i.e.: ΔOD_{def} = optical density difference between 2 and 22 hrs of defaunated parallels (treated with 200 mg/mL of the specific eukaryotic inhibitor digitonin).

41. To compensate for unspecific optical density changes the corrected mean OD- difference for all replicates of the control and treatment groups is calculated as:

$$\Delta OD_{corr} = \Delta OD - \text{mean } \Delta OD_{def}$$

where:

ΔOD: ΔOD-value of a single sample without digitonin (6 controls and 3 samples per chemical concentration);

meanΔOD_{def}: mean ΔOD-value of the (defaunated) phagocytosis-free, digitonin-containing control (n=6) and the respective digitonin-containing chemical treatment group (n=3).

Test performance

42. The percentage decrease of the OD of controls (important to validity, see para 9) is calculated as:

$$\%\Delta OD(\text{control}) = \frac{\text{mean } \Delta OD_{corr}(\text{controls})}{\text{mean 2h-OD(controls)}} \times 100$$

where:

mean ΔOD_{corr}(controls): mean ΔOD(controls) – mean ΔOD_{def} (controls); n=6;

mean 2h-OD(control): mean of the 2h-OD (start-OD at t1=2hrs) for all control replicates (n=6).

43. The percentage 'unspecific' decrease of the OD for phagocytosis-free. digitonin-containing controls (important to validity, see para 9) is calculated as:

$$\%\Delta OD_{def}(\text{controls}) = \frac{\text{mean } \Delta OD_{def}(\text{controls})}{\text{mean 2h-OD}_{def}(\text{controls})} \times 100$$

where:

mean ΔOD_{def}(controls): mean of the OD-difference between 2hrs and 22hrs for replicates of digitonin controls (n=6);

mean 2h-OD_{def}(controls): mean of the 2h-OD (start-OD at t1=2hrs) for replicates of digitonin controls (6 replicates).

44. The calculation of chemical effects on the optical density (important to validity. see para 9) is made according to:

$$\%\ \Delta OD_{def}(\text{treatment}) = \frac{\text{mean}\ \Delta OD_{def}(\text{treatment})}{\text{mean 2h-}OD_{def}(\text{treatment})} \times 100$$

where:

mean ΔOD_{def}(treatment): mean of the OD-difference between 2hrs and 22hrs for replicates of a treatment group (n=3) containing digitonin;

mean 2h-OD_{def}(treatment): mean of the 2h-OD (start-OD at t1=2hrs) for replicates of a treatment group (3 replicates each) containing digitonin.

<u>Reduction of phagocytosis</u>

45. The inhibition of the phagocytotic activity for each replicate at each substance concentration is expressed as a percentage of the mean of the control phagocytotic activities:

$$\%\text{Inhibition}_x = 1 - \frac{\Delta OD_{corr}(\text{replicate}_x \text{ of treatment group})}{\text{mean}\Delta OD_{corr}(\text{controls})} \times 100$$

where:

ΔOD_{corr}(replicate$_x$ of treatment group):corrected OD-difference between measurements after 2hrs and 22hrs for the respective replicate (vial) of the chemical concentration (treatment group), i.e. ΔOD_{corr}(replicate$_x$ of treatment group) = ΔOD(replicate$_x$ of treatment group) - meanΔOD_{def}(replicates$_{1-3}$ of the respective treatment containing digitonin);

meanΔOD_{corr}(controls): mean value for corrected OD-differences between controls and controls containing digitonin, i.e. mean of ΔOD(controls) - meanΔOD_{def}(controls).

When solvents are used to prepare the test solutions, the solvent controls rather than the controls without solvents should be used in calculation of percent inhibition.

<u>Plotting concentration response curves</u>

46. Plot the percentage of inhibition for each individual vial (replicate) against the logarithm of the test substance concentration (n=3 for each concentration), compare inhibition curve, APPENDIX VIII - Concentration-response curves.

47. Include the data of the unspecific OD-change of defaunated parallels for each replicate (n=3 for each concentration) according to:

$$\% \Delta OD_{def}(replicate_x) = \frac{\Delta OD_{def}(replicate_x)}{\text{mean 2h-}OD_{def}(treatment)} \times 100;$$

see also inhibition curve, Figure 17 of Appendix VIII - .

Evaluation of EC-values

48. EC_X-values are derived by statistical means. All data are normalized by dividing by corresponding data for the controls (average of all control replicates). Concentration-response curves are fitted with nonlinear regression methods on log-transformed concentrations. Calculations of preferably EC_{50}-values should be performed on the basis of three- and four- parameter (symmetrical and asymmetrical) sigmoidal functions. Each replicate is treated as a separate point. The top and bottom plateau are constrained to 0% and 100%, respectively (some substances may stimulate the growth at low concentrations, referred to as hormesis. Only data points indicating effects between 0 and 100% should be considered). EC-values should be reported with 95% confidence limits.

49. Where the data obtained are inadequate for the use of standard curve-fitting methods of calculating the EC_{50}, the highest concentration causing no effect and the lowest concentration producing 100 per cent inhibition should be used as an approximation for the EC_{50} (this being considered the geometric mean of these two concentrations).

Test report

50. The test report must include the following information:

Test substance

- identification data
- purity, physical nature and. where relevant physicochemical properties

Activated sludge

- origin, conditions of operation of the wastewater treatment plant and influent it receives, dry weight, day of sampling, storage conditions (if relevant), any pretreatment etc.

Test conditions:

- date of the start and the end of the test
- activated sludge concentration (mixed liquor suspended solids)
- temperature
- bacterial strain (culturing. source/origin)
- test vessel and apparatus
- vehicle and method used for solubilizing the test substance and concentration of the vehicle in the test solutions
- concentrations tested (measured or nominal)
- information of concentrations of test substances in the test solutions, analytical method

Results:

- optical density values for each vessel at each measuring point
- mean values of replicates

- graphical presentation of the concentration effect relationships
- EC_{50}-values with 95% confidence limits and method of calculation
- other observed effects, incidents which might have influenced the results

11 APPENDIX V - Substrate

Substrate

Commercially available lyophilized cells of the *E.coli*-strains ATCC 9637, ATCC 8739 and K12 (Sigma-Aldrich) have been found to meet the requirements of a low tendency to aggregate, to flocculate and to adhere to activated sludge flocs. They are also suitable as food source for phagotrophic sludge inhabitants.

Additionally it has been shown for both ATCC strains that an appropriate substrate can also be produced by cultivating and subsequent lyophilisation of cells: a spatula tip of *E. coli* powder was added to 2 mL Terrific broth medium (TB) in a 12-mL glass tube with metal cap and shaken overnight in an incubator. 37°C at 200 rpm. 100 µL-aliquots were mixed with glycerine (final concentration 15%) and stored at -80°C. 5 µL of this *E. coli* stock culture were used as inoculum of 200 or 500 mL-Erlenmeyer flasks containing 50 or 100 mL TB, respectively. The cultures were incubated at 37°C and continuously shaken for aeration (200 rpm). After 16-18 h. the early stationary-phase cells were spun down ($10^4 \times g$), washed in 0.9% (w/v) NaCl and lyophilized, i.e the cell mass was frozen at -80°C and the remaining fluid vaporized under vacuum in a lyophilisator (Alpha 1-4 LD, Martin Christ). It should, however, be noted, that not all preparations showed satisfactory results[25] and that each preparation has to be examined with regard to a low binding of the bacterial substrate to sludge flocs under testing conditions.

Other bacterial strains are of course not excluded if they fulfill the validity criteria.

[25] It is known that environmental conditions and the bioavailability of nutrients may considerably shift the adhesive properties of bacterial cells (e.g. Faille, et al., 2002; Bonaventura, et al., 2008) and that bacterial strains have quite considerable possibilities of hydrophobic property variations in the course of growth (e.g. Jorand, et al., 1994).

12 APPENDIX VI - Test medium

Test medium

Test medium: moderately hard EPA synthetic water (EPA, 2002), pH-buffered

I. preparation of chemical stock solutions

1) prepare 100 mL of a 40-fold concentrated stock solution for each reagent 1-5:

reagent	final conc. in test	conc. factor	reagent per 100 mL		
(1) NaHCO3	96 mg/L	40x	384 mg	ad 100 mL H2O	
(2) CaSO4 · 2H2O	60 mg/L	40x	240 mg	ad 100 mL H2O	
(3) MgSO4	60 mg/L	40x	240 mg	ad 100 mL H2O	
(4) KCl	4 mg/L	40x	16 mg	ad 100 mL H2O	
(5) Hepes, pH 7.5	10 mM	40x	9.532 g	adjust pH	ad 100 mL H_2O

2) adjust the pH of the HEPES buffer-solution to pH 7.5 (NaOH)

a) dissolve 9.532 g HEPES reagent in ca. 90 mL of H_2O

b) adjust the pH to 7.5 with a concentrated NaOH solution (ca. 2.8 mL of a 5 M (200g/L) NaOH solution)

c) fill up to 100 mL with deion. (or distilled) water

3) store the test medium stocks at -20°C (for practical reasons it is recommended to aliquote the stocks)

II. preparation of the standard moderately hard EPA synthetic water, pH-buffered

1) Fill a 200 milliliter volumetric flask with approximately 160 ml deionized (or distilled) water and

2) add 5 mL of each concentrated stock solution, in the sequence 1 to 5.

3) Add deionized (or distilled) water up to the 200 ml mark and shake to homogenize the medium.

→ store the (diluted) test medium at 4-6°C for no longer than 2 weeks.

13 APPENDIX VII - Preparation of digitonin solution

Digitonin (CAS Number 11024-24-1) tends to *form precipitates* in solution, but it dissolves readily in boiling water. After cooling the solution remains clear for several hours.

Add 100 mg digitonin to 10 mL H_2O (= 10 g/L = 50-fold concentrated solution), heat the solution to about 95°C-100°C for 5 minutes and vortex slowly to dissolve the precipitate. Cool to room temperature prior to use (*Note*: Commercial digitonin powder is a mixture consisting of about five glycosides (Fukunaga, et al., 1988). One of the main components is digitonin, which amounts to about 50% of the preparation (TLC) from Sigma-Aldrich used in the ring study).

14 APPENDIX VIII - Concentration-response curves

Concentration-response curves

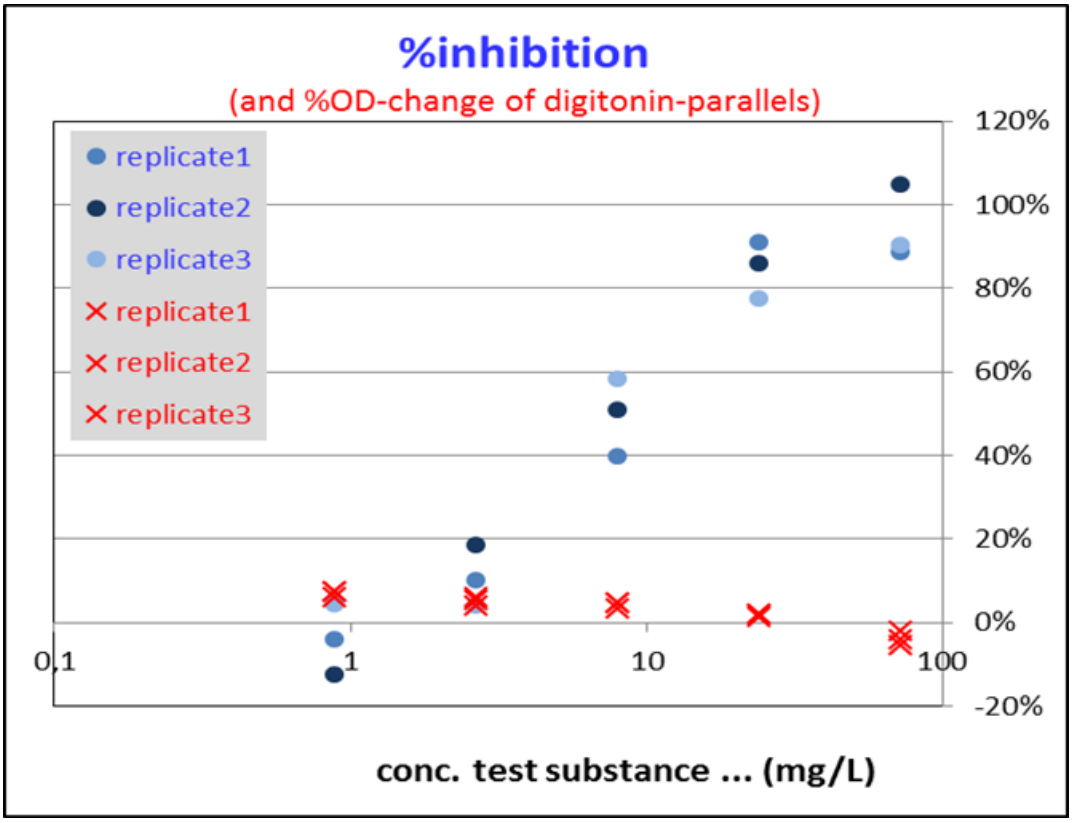


Figure 17: Exemplary concentration-response plot with %inhibition data (triplicates) for the phagocytosis activity (filled circle) and the %change of the defaunated digitonin-triplicates (cross).

15 APPENDIX IX - OD vs. *E.coli* concentration

Relationship between OD_{440} nm and *E. coli* concentration

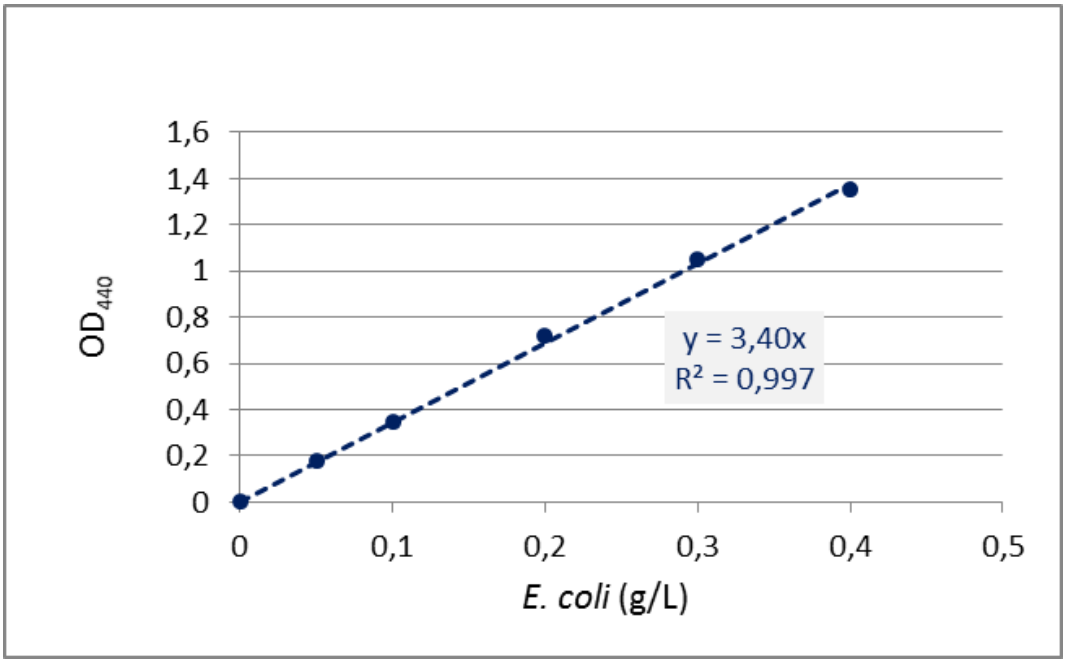


Figure 18: Plot of optical density at λ = 440 nm versus the concentration of the *E. coli* strain ATCC9637.

16 LITERATURE

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