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Effect studies of new hormone active substances on aquatic snails *Potamopyrgus antipodarum*

Final report

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Effect studies of new hormone active substances on aquatic snails *Potamopyrgus antipodarum*

Final report

by

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Abstract: Effect studies of new hormone active substances on aquatic snails Potamopyrgus antipodarum

In the recent study, two endocrine active substances for human use, the progestin Dienogest and the glucocorticoid Dexamethasone, were tested in a 28-day reproduction test with the New Zealand mud snail *Potamopyrgus antipodarum* according to the OECD guideline 242. In a ring test in 2016, in which the Fraunhofer IME successfully participated, the OECD test guideline 242 was validated in order to broaden the range of test methods for investigations of e.g. endocrine active substances and their endocrine and reproductive toxic effects on aquatic invertebrates, particularly on water snails as they are proved to be sensitive towards endocrine disruptors (EDs). Until then the focus in testing of EDs was mainly on studies with fish. The results of this study will be used to establish a tailored evaluation concept for environmental risks for progestins and artificial glucocorticoids.

In the reproduction test with Dienogest analytically verified concentrations of 6.97, 11.2, 25.6, 106 and 332 ng/L were introduced. After 28 days, no significant effect on any of the investigated endpoints was observed. The NOEC for reproduction (in terms of total, shelled and unshelled embryos), survival and length were therefore determined to be ≥ 332 ng/L, respectively.

The reproduction test with Dexamethasone was performed with analytically verified concentrations of 1.06, 3.54, 10.5, 36.3 and 105 µg/L. After 28 days, no significant effect on reproduction, survival and length occurred, resulting in a NOEC of ≥ 105 µg/L, respectively.

Kurzbeschreibung: Studien zur Untersuchung der Wirkung neuer, hormonaktiver Wirkstoffe auf Wasserschnecken *Potamopyrgus antipodarum*

In der vorliegenden Studie wurden zwei Humanarzneimittel mit hormonaktiver Wirkung – das Gestagen Dienogest und das Glococortikoid Dexamethason – in einem 28-tägigen Reproduktiontest mit der Neuseeländischen Zwerdeckelschnecke *Potamopyrguas antipodarum* nach OECD Richtlinie 242 untersucht. In einem Ringtest im Jahr 2016, an dem das Fraunhofer IME erfolgreich teilgenommen hat, wurde die OECD Richtlinie 242 validiert. Die Validierung erfolgte besonders im Hinblick auf die Erweiterung des Spektrums von Testmethoden für die Untersuchung von endokrin wirksamen Substanzen und deren endokrin-disruptiven und reproduktionstoxischen Wirkungen auf aquatische Invertebraten, insbesondere auf Wasserschnecken, da diese als sensitiv gegenüber endokrinen Disruptoren (EDs) gelten. Zuvor lag der Schwerpunkt in der Untersuchung von EDs vorwiegende auf Fischen als Testorganismen. Aus den Ergebnissen der vorliegenden Studie soll ein maßgeschneidertes Bewertungskonzept für Umweltrisiken für Gestagene und synthetische Glucocortikoide abgeleitet werden.

Im Reproduktionstest mit Dienogest wurden analytisch gemessene Konzentrationen von 6.97, 11.2, 25.6, 106 und 332 ng/L eingesetzt. Nach 28 Tagen wurde kein signifikanter Effekt auf die betrachteten Endpunkte Reproduktion (Gesamtembryonen, beschalte Embryonen und unbeschalte Embryonen), Überleben und Länge an Testende beobachtet. Die NOEC für alle betrachteten Endpunkte wurde somit auf ≥ 332 ng/L festgelegt.

Im Reproduktionstest mit Dexamethason wurden analytisch gemessene Konzentrationen von 1.06, 3.54, 10.5, 36.3 und 105 µg/L getestet. Nach 28 Tagen wurde kein signifikanter Effekt auf die Reproduktion sowie auf das Überleben und die Länge an Testende beobachtet. Die NOEC der betrachteten Endpunkte wurde somit auf ≥ 105 µg/L festgelegt.

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ANOVA	Analysis of Variance
CPA	Cyproteron acetate
DOC	Dissolved Organic Carbon
EAS	Endocrine active (pharmaceutical) substances
$EC_{10/20/50}$	Effective concentration: Is the concentration of the test item, which results in a 10, 20 or 50 percent change (inhibition or enhancement) in the measured parameter relative to the control
EDs	Endocrine Disruptors / Endocrine Disrupting chemicals
ESI	Electrospray Ionization
FKZ	Forschungskennziffer (engl.: Project number for the research project)
IS	Internal Standard
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LOEC	Lowest observed effect concentration: Is the lowest concentration at which the measured parameter shows significant change relative to the control.
LOQ	Limit of Quantification
MS	Mass Spectrometry
MRM	Multiple Reaction Monitoring
$NaHCO_3$	Sodium hydrogen carbonate
n.d.	not determined
NOEC	No observed effect concentration: Is the highest concentration tested at which the measured parameter shows no significant change relative to the control.
OECD	Organization for Economic Co-operation and Development
RSD	Relative Standard Deviation
SD	Standard Deviation
TWM	Time Weighted Mean
UPLC	Ultra Performance Liquid Chromatography
ZEOGRT	Zebrafish One Generation Reproduction Test

Summary

In the last decades it has become clear that both humans and the environment - in particular the aquatic environment - are increasingly exposed to the influences of hormone-like substances. This is reflected in increasing numbers of hormonal diseases in humans or growth disturbance and reduced reproductive performance in organisms in the environment. Many of the observed effects are attributed to chemicals known as endocrine disruptors (EDs). EDs are hormone-active substances that can cause changes in the hormonal system even in minor concentrations. In the scientific literature, there are some studies dealing with specific effects of endocrine active substances (EAS) on aquatic organisms but these findings are mostly related to fish. Therefore, in the future the focus on other species such as aquatic invertebrates must be increased.

In 2016, an OECD guideline (OECD TG 242) with the New Zealand mud snail *Potamopyrgus antipodarum*, was developed to investigate endocrine and reproductive toxic effects on water snails. In the chronic snail test, female snails are exposed to a test substance for 28 days. Reproduction, survival and length of test organisms at test end are evaluated as endpoints.

The aim of the project "Effect studies of new hormone active substances on aquatic snails *Potamopyrgus antipodarum*" was to gain new insights into the effects of progestins and glucocorticoids on aquatic invertebrates, in particular water snails. For this, two medicinal substances for human use were tested, the progestin Dienogest (CAS No. 65928-58-7) and the glucocorticoid Dexamethasone (CAS No. 50-05-2). The influence of both substances on the reproduction of water snails of the species *Potamopyrgus antipodarum* was investigated using the 28-day reproduction test according to OECD 242.

The results will be used to establish a tailored evaluation concept for environmental risks for progestins and glucocorticoids.

Material and Methods

Literature research

In the first part of the project, a literature research has been performed in order to summarize information on effects of different progestins and glucocorticoids on invertebrates, or snails in particular. The research should help to identify an appropriate concentration range for the reproduction tests. For the research mainly open access databases were used (PubMed, ScienceDirect, Web of Science, Google Scholar). Information on test substance, test organism, duration of the test, observed endpoints and effect concentrations were collected and summarized in a tabular form.

General performance of the reproduction test with Potamopyrgus antipodarum

In the OECD 242 reproduction test, adult female snails of the species *Potamopyrgus antipodarum* were exposed to five concentrations of the test item for 28 days. Each treatment group consisted of six replicates, respectively. Each of the six replicates contained six snails (=36 snails per treatment). Untreated control replicates were run in parallel. The test item was dissolved in reconstituted water and adult snails were subsequently introduced into the test vessels. The test was performed under semi-static exposure conditions with three times media renewals per week. At the end of the test, reproduction, survival and length of *P. antipodarum* at the end of the exposure period were examined. The reproduction of the snails was evaluated by counting embryo numbers in the brood pouch of the test organisms at test end. The total number of embryos was counted and, in addition, it was differentiated if the embryos were shelled or

unshelled. Effects on reproductive performance, survival and growth (adults length at test termination) were investigated using appropriate statistical methods.

The No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) were determined by one-way ANOVA, followed by an appropriate post-hoc test.

During both tests, chemical analysis was performed in weekly intervals to monitor the actual exposure concentrations using a LC-MS/MS system. In addition, concentrations of each treatment level were measured at the beginning and end of each test

The tests were performed according to OECD 242 (5).

Results and Discussion

Literature research

The literature research on Dienogest provided 13 references in total, of which four references were considered as relevant for the determination of the final concentration range for the reproduction test. In contrast to that, no relevant references were found for Dexamethason.

The goal of the study was to demonstrate that the snail *Potamopyrgus antipodarum* is less sensitive against the progestin Dienogest and the glucocorticoid Dexamethasone compared to the zebrafish *Danio rerio* which was used in a fish study (ZEOGRT) with Dienogest (FKZ 3717 57 405 0) before. Based on this and on the results of the literature research the concentration ranges for the reproduction tests were determined.

The nominal concentration range for Dienogest was determined to 3.20, 10.0, 32.0, 100 and 320 ng/L.

For Dexamethasone, a nominal concentration range of 1.00, 3.20, 10.0, 32.0 and 100 µg/L was selected.

For both concentrations ranges the maximum allowed spacing factor of 3.16 provided in the guideline was used.

Reproduction test with Potamopyrgus antipodarum

Progestin Dienogest

In the reproduction test with Dienogest, the actual exposure concentrations of the test item in the aqueous test media were determined by LC-MS/MS (LOQ= 1.5 ng/L). Except of the first concentration, which ranged from 208 – 228 % of nominal, measured test item concentrations were between 78.8 and 115 % of nominal concentrations in fresh samples and 73.1 and 113 % in aged samples throughout the test. The deviation of the measured test item concentrations from the nominal test concentrations was greater than 20%. However, concentrations remained stable within ± 20 % of measured initial concentrations (85 – 105% of initial). The results were based on the geometric mean measured test item concentrations of 6.97, 11.2, 25.6, 106 and 332 ng/L (218, 112, 80.1, 106 and 104 % of nominal concentrations).

No snail died during the test neither in the control nor in any treatment. Mean length of control snails was 3.61 mm and mean length of snails in treatments ranged from 3.58 to 3.61 mm. Compared to the control, no significant effect on survival or growth were observed, resulting in a NOEC of ≥ 332 ng/L for both endpoints.

At test end, the total number of embryos ranged from 7.18 to 9.32 across all treatments and controls. The number of shelled embryos and unshelled embryos ranged from 3.58 to 4.43 and 3.65 to 4.85 embryos, respectively. No significant effect on the total number of embryos and on the number of unshelled or shelled embryos was observed at the end of the exposure period

compared to the control. Thus, based on geometric mean measured concentrations, for reproduction a NOEC of ≥ 332 ng/ was identified.

A summary of determined NOECs and LOECs for reproduction, survival and length is given in Table 1.

Table 1: Summary of the effect concentrations based on geometric mean measured test item concentrations in the snail reproduction test with Dienogest given in ng/L.

Parameter	EC50	EC10	NOEC	LOEC
Total number of embryos	n.d.	n.d.	≥ 332	> 332
Number of unshelled embryos	n.d.	n.d.	≥ 332	> 332
Number of shelled embryos	n.d.	n.d.	≥ 332	> 332
Survival	n.d.	n.d.	≥ 332	> 332
Growth	n.d.	n.d.	≥ 332	> 332

n.d. = not determined

Glucocorticoid Dexamethasone

In the reproduction test with Dexamethasone, the actual exposure concentrations of the test item in the aqueous test media were determined by LC-MS/MS (LOQ= 0.1 µg/L). Measured test item concentrations were between 99.7 and 116 % of nominal concentrations in fresh samples and between 96.6 and 120 % in aged samples throughout the test. The deviation of the measured test item concentrations from the nominal test concentrations was within ± 20%. In addition, concentrations remained stable within ± 20 % of measured initial concentrations (91.0 – 117 % of initial). The results were based on the geometric mean measured test item concentrations of 1.06, 3.54, 10.5, 36.3 and 105 µg/L (106, 111, 105, 114 and 105 % of nominal concentrations).

No snail died during the test neither in the control nor in any treatment. Mean length of control snails was 3.76 mm and mean length of snails in treatments ranged from 3.69 to 3.78 mm. Compared to the control, no significant effect on survival or growth were observed, resulting in a NOEC of ≥ 105 µg/L for both endpoints.

At test end, the total number of embryos ranged from 12.4 to 15.8 across all treatments and the control. The number of shelled embryos and unshelled embryos ranged from 5.80 to 8.55 and 6.38 to 8.15 embryos, respectively. No significant effect on the total number of embryos and on the number of unshelled embryos was observed at the end of the exposure period compared to the controls. Thus, based on geometric mean measured concentrations, for reproduction of total and unshelled embryos a NOEC of ≥ 105 µg/L was identified, respectively. Contrarily, a significant decrease in the number of shelled embryos was observed for each treatment level, resulting in a NOEC of < 1.06 µg/L. However, according to the guideline OECD 242, the relevant parameter for calculation of reproductive effect concentrations is the total number of embryos, which showed no test item related effect. Therefore, the NOEC derived for reproduction in this study was ≥ 105 µg/L.

A summary of determined NOECs and LOECs for reproduction, survival and length is given in Table 2.

Table 2: Summary of the effect concentrations based on geometric mean measured test item concentrations in the snail reproduction test with Dexamethasone given in µg/L.

Parameter	EC50	EC10	NOEC	LOEC
Total number of embryos	n.d.	n.d.	≥ 105	> 105
Number of unshelled embryos	n.d.	n.d.	≥ 105	> 105
Number of shelled embryos	n.d.	n.d.	< 1.06	≤ 1.06
Survival	n.d.	n.d.	≥ 105	> 105
Growth	n.d.	n.d.	≥ 105	> 105

n.d. = not determined

Conclusion

In a 28-day reproduction test according to OECD Guidance 242 (5) the toxicity of the progestin Dienogest and the glucocorticoid Dexamethasone was investigated.

In the reproduction test with Dienogest, snails were exposed to geometric mean measured concentrations of 6.97, 11.2, 25.6, 106 and 332 ng/L. After 28 days, no significant difference was observed between control and any treatment regarding the investigated parameters reproduction (in terms of total number of embryos, shelled and unshelled embryos), growth and mortality. Therefore, the NOEC for each endpoint was set to be ≥ 332 ng/L.

The progestin Dienogest therefore has no toxic effect on reproduction of the water snail *Potamopyrgus antipodarum* up to a concentration of 332 ng/L.

In the reproduction test with Dexamethasone, snails were exposed to geometric mean measured concentrations of 1.06, 3.54, 10.5, 36.3 and 105 µg/L. After 28 days, no significant difference was observed between control and any treatment regarding the investigated parameters reproduction (in terms of total number of embryos and unshelled embryos), growth and mortality. Therefore, the NOEC for these endpoints was set to be ≥ 105 µg/L. Contrarily, a significant reduction of shelled embryo numbers was observed in each treatment, resulting in a NOEC < 1.06 µg/L for shelled embryos. According to the guideline OECD 242, the relevant parameter for calculation of reproductive effect concentrations is the total number of embryos, which showed no test item related effect. Therefore, the NOEC derived for reproduction in the performed study was ≥ 105 µg/L.

Thus, the glucocorticoid Dexamethasone has no toxic effect on reproduction of the water snail *Potamopyrgus antipodarum* up to a concentration of 105 ng/L.

Zusammenfassung

In den letzten Jahrzehnten hat sich gezeigt, dass sowohl Menschen als auch die Umwelt - insbesondere die aquatische Umwelt - zunehmend den Einflüssen hormonähnlicher Substanzen ausgesetzt sind. Dies spiegelt sich bei Menschen in einer zunehmenden Anzahl von hormonellen Erkrankungen wieder während es in der Umwelt zu Wachstumsstörungen und verminderter Fortpflanzungsleistung kommen kann. Viele der beobachteten Effekte werden auf Chemikalien zurückgeführt, die als endokrine Disruptoren (EDs) bezeichnet werden. EDs sind hormonaktive Substanzen, die bereits in geringsten Konzentrationen Veränderungen im Hormonsystem bewirken können. In der wissenschaftlichen Literatur gibt es einige Studien, die sich mit den spezifischen Auswirkungen endokrin aktiver Substanzen (EAS) auf Wasserorganismen befassen. Diese Studien beziehen sich jedoch hauptsächlich auf Fische. Daher muss in Zukunft der Fokus auf andere Arten wie z.B. aquatische Invertebraten verstärkt werden.

Im Jahr 2016 wurde eine OECD-Richtlinie (OECD TG 242) mit der Neuseeländischen Zwergdeckelschnecke *Potamopyrgus antipodarum* entwickelt, um endokrine und reproduktionstoxische Effekte auf Wasserschnecken zu untersuchen. Im chronischen Schneckentest werden weibliche Schnecken 28 Tage lang einer Testsubstanz ausgesetzt. Die Reproduktion, Überlebensrate und die Länge der Organismen an Testende werden als Endpunkte bewertet.

Ziel des vorliegenden Projektes war es, neue Erkenntnisse über die Wirkung von Gestagenen und Glucocortikoiden auf aquatische Invertebraten, insbesondere auf Wasserschnecken, zu gewinnen. Dazu wurden zwei Humanarzneimittel, das Gestagen Dienogest (CAS-Nr. 65928-58-7) und das Glucocortikoid Dexamethason (CAS-Nr. 50-05-2) als Testsubstanzen ausgewählt. Der Einfluss beider Substanzen auf die Reproduktion von Wasserschnecken der Art *Potamopyrgus antipodarum* wurde jeweils in einem 28-tägigen Reproduktionstest nach OECD 242 untersucht.

Aus den Ergebnissen soll ein maßgeschneidertes Bewertungskonzept für Umweltrisiken für Gestagene und Glucocortikoide erstellt werden.

Material und Methoden

Literaturrecherche

Im ersten Teil des Projekts wurde eine Literaturrecherche durchgeführt, um Informationen über die Auswirkungen von Gestagenen und Glucocortikoiden auf Invertebraten, insbesondere auf Wasserschnecken, zu sammeln. Die Literaturrecherche sollte dabei helfen einen geeigneten Konzentrationsbereich für die Reproduktionstests zu ermitteln. Für die Recherche wurden öffentliche Datenbanken wie PubMed, ScienceDirect, Web of Science oder Google Scholar verwendet. Aus den Suchabfragen wurden Informationen über Prüfsubstanz, Testorganismen, Dauer der Tests, betrachtete Endpunkte und Wirkkonzentrationen gesammelt und in tabellarischer Form zusammengefasst.

Durchführung des Reproduktionstestes mit Potamopyrgus antipodarum

Im Reproduktionstest nach OECD 242 wurden adulte, weibliche Schnecken der Art *Potamopyrgus antipodarum* über 28 Tage fünf Konzentrationen der jeweiligen Testsubstanz exponiert. Jede Behandlungsstufe bestand aus jeweils sechs Replikaten mit jeweils sechs Schnecken pro Replikat (=36 Schnecken/Konzentration). Parallel dazu wurden unbehandelte Kontrollreplikate angesetzt. Die Testsubstanz wurde in synthetischem Medium nach OECD 242 gelöst und anschließend wurden die adulten Schnecken in die mit Testlösung gefüllten Glasgefäße eingesetzt. Der Test wurde unter semi-statischen Bedingungen mit dreimaligem Mediumwechsel pro Woche durchgeführt. Am Ende des Tests wurde die Reproduktion, die

Überlebensrate und die Länge der Schnecken an Testende bestimmt. Die Reproduktion der Schnecken wurde durch Auszählung der Embryonen in der Bruttasche jeder einzelnen Schnecke bewertet. Dabei wurde sowohl die Gesamtzahl als auch die Anzahl der unbeschalten und beschalten Embryonen bestimmt. Mögliche Effekte auf Reproduktion, Überleben und Wachstum wurden mit Hilfe geeigneter statistischer Methoden untersucht.

Die No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) wurden durch einseitige ANOVA gefolgt von geeigneten Post-Hoc Tests berechnet.

Während beider Tests wurde in der niedrigsten und in der höchsten Testkonzentration in wöchentlichen Abständen eine chemische Analytik durchgeführt, um die tatsächlichen Expositionskonzentrationen zu bestimmen. Die Bestimmung erfolgte mit einem LC-MS/MS Messsystem. Zusätzlich wurden jeweils an Teststart und Testende Proben aus allen Testkonzentrationen analytisch quantifiziert.

Beide Tests wurden nach OECD 242 durchgeführt (5).

Ergebnisse und Diskussion

Literaturrecherche

Die Literaturrecherche zu Dienogest lieferte insgesamt 13 Referenzen, von denen vier Referenzen als relevant für die Bestimmung des finalen Konzentrationsbereichs für den Reproduktionstest erachtet wurden. Im Gegensatz dazu wurden für Dexamethason keine relevanten Literaturstellen gefunden.

Ziel des Gutachtens war es, die Sensitivität von *Potamopyrgus antipodarum* gegenüber Dienogest und Dexamethason im Vergleich zum Zebrafisch (*Danio rerio*), der zuvor als Testorganismus in einer Fischstudie (ZEOGRT) mit Dienogest (FKZ 3717 57 405 0) eingesetzt wurde, zu untersuchen. Aus diesem Grund und basierend auf den Ergebnissen der Literaturrecherche wurden folgende Konzentrationsbereiche für die Reproduktionstests festgelegt:

Der nominale Konzentrationsbereich für den Reproduktionstest mit Dienogest wurde auf 3.20, 10.0, 32.0, 100 und 320 ng/L festgelegt.

Für den Reproduktionstest mit Dexamethason wurde ein nominaler Konzentrationsbereich von 1.00, 3.20, 10.0, 32.0 und 100 µg/L bestimmt.

Für beide Konzentrationsbereiche wurde der in der Richtlinie vorgegeben maximale Separationsfaktor von 3.16 verwendet.

Reproduktionstest mit Potamopyrgus antipodarum

Gestagen Dienogest

Im Reproduktionstest mit Dienogest wurden die Testkonzentrationen der Testsubstanz im wässrigen Medium mit einer LC-MS/MS Methode gemessen (LOQ = 1.5 ng/L). Mit Ausnahme der niedrigsten Behandlungsstufe, für die die Wiederfindungen der gemessenen Konzentrationen in einem Bereich von 208 – 228 % der nominalen Konzentrationen lagen, lagen die gemessenen Konzentrationen der Testsubstanz während des gesamten Tests zwischen 78.8 und 115% der nominalen Konzentrationen. Dabei wurden für die frischen Testlösungen Wiederfindungen von 78.8 – 115 % und für die gealterten Testlösungen Wiederfindungen zwischen 73.1 und 113% bestimmt. Die Abweichung der gemessenen Konzentrationen von den nominalen Konzentrationen war somit größer als 20%. Die gemessenen Konzentrationen der gealterten Testlösungen blieben innerhalb von ± 20% bezogen auf die inital gemessenen Konzentrationen der frischen Testlösungen (85 – 105% der Anfangskonzentrationen) und können somit als stabil betrachtet werden. Der Test wurde auf die geometrisch gemittelten, gemessenen

Konzentrationen von 6.97, 11.2, 25.6, 106 und 332 ng/L (218, 112, 80.1, 106 und 104 % der nominalen Konzentrationen) ausgewertet.

Während der 28-tägigen Exposition starben keine Testorganismen, weder in der Kontrolle noch in den Behandlungsstufen. Die mittlere Länge der Kontrollschnecken an Testende betrug 3.61 mm und die mittlere Länge der Schnecken in allen Behandlungsstufen lag zwischen 3.58 und 3.61 mm. Im Vergleich zur Kontrolle wurden keine signifikanten Unterschiede für Überleben und Wachstum festgestellt, wodurch sich für beide Endpunkte eine NOEC von ≥ 332 ng/L ergibt.

Am Ende des Tests lag die Gesamtzahl der Embryonen zwischen 7.18 und 9.32 über alle Behandlungsstufen und die Kontrolle. Die Anzahl der beschalten und unbeschalten Embryonen lag bei 3.58 – 4.43 beschalten Embryonen und 3.65 – 4.85 unbeschalten Embryonen. Am Ende der Expositionszeit wurde im Vergleich zur Kontrolle kein Einfluss der Testsubstanz auf die Gesamtzahl der Embryonen sowie auf die Anzahl der beschalten und unbeschalten Embryonen festgestellt. So wurde basierend auf den analytisch verifizierten Konzentrationen für die Reproduktion eine NOEC von ≥332 ng/L abgeleitet.

Eine Zusammenfassung der bestimmten NOECs und LOECs für die Endpunkte Reproduktion, Überleben und Wachstum ist in Table 3 dargestellt.

Table 3: Zusammenfassung der Effektkonzentrationen basierend auf den geometrisch gemittelten, gemessenen Testkonzentrationen im Schnecken Reproduktionstest mit Dienogest in ng/L.

Parameter	EC50	EC10	NOEC	LOEC
Gesamtanzahl der Embryonen	n.d.	n.d.	≥ 332	> 332
Anzahl unbeschalter Embryonen	n.d.	n.d.	≥ 332	> 332
Anzahl beschalter Embryonen	n.d.	n.d.	≥ 332	> 332
Überleben	n.d.	n.d.	≥ 332	> 332
Wachstum	n.d.	n.d.	≥ 332	> 332

n.d. = nicht bestimmt

Glucocortikoid Dexamethason

Im Reproduktionstest mit Dexamethason wurden die Testkonzentrationen der Testsubstanz im wässrigen Medium mit einer LC-MS/MS Methode überprüft (LOQ = 0.1 µg/L). Die gemessenen Konzentrationen der Testsubstanz lagen während des gesamten Tests zwischen 96.6 und 120% der nominalen Konzentrationen. Dabei wurden für die frischen Testlösungen Wiederfindungen von 99.7 – 116 % und für die gealterten Testlösungen Wiederfindungen zwischen 96.6 und 120 % bestimmt. Die Abweichung der gemessenen Konzentrationen von den nominalen Konzentrationen war somit kleiner als 20%. Zudem blieben die gemessenen Konzentrationen der gealterten Testlösungen innerhalb von ± 20% bezogen auf die initial gemessenen Anfangskonzentrationen der frischen Testlösungen (91.0 – 117% der Anfangskonzentrationen) und können somit als stabil betrachtet werden. Der Test wurde auf die geometrisch gemittelten, gemessenen Konzentrationen von 1.06, 3.54, 10.5, 36.3 und 105 µg/L (106, 111, 105, 114 und 105 % der nominalen Konzentrationen) ausgewertet.

Während der 28-tägigen Exposition starben keine Testorganismen, weder in der Kontrolle noch in den Behandlungsstufen. Die mittlere Länge der Kontrollschnecken am Testende betrug 3.76 mm und die mittlere Länge der Schnecken in allen Behandlungsstufen lag zwischen 3.67 und 3.78 mm. Im Vergleich zur Kontrolle wurden keine signifikanten Unterschiede für

Überleben und Wachstum festgestellt, wodurch sich für beide Endpunkte eine NOEC von ≥ 105 µg/L ergibt.

Am Ende des Tests lag die Gesamtzahl der Embryonen zwischen 12.4 und 15.8 über alle Behandlungsstufen und Kontrolle. Die Anzahl der beschalten und unbeschalten Embryonen lag bei 5.80 – 8.55 beschalten Embryonen und 6.38 – 8.15 unbeschalten Embryonen. Am Ende der Expositionszeit wurde im Vergleich zur Kontrolle kein Einfluss der Testsubstanz auf die Gesamtzahl der Embryonen sowie auf die Anzahl der unbeschalten Embryonen festgestellt. So wurde basierend auf den analytisch verifizierten Konzentrationen eine NOEC von ≥ 105 µg/L abgeleitet. Im Gegensatz dazu wurde allerdings eine signifikante Abnahme der beschalten Embryonen in allen Testkonzentrationen festgestellt. Die entsprechende NOEC wurde daher auf < 1.06 µg/L festgelegt.

Eine Zusammenfassung der bestimmten NOECs und LOECs für die Endpunkte Reproduktion, Überleben und Wachstum ist in Table 4 dargestellt.

Table 4: Zusammenfassung der Effektkonzentrationen basierend auf den geometrisch, gemittelten gemessenen Testkonzentrationen im Schnecken Reproduktionstest mit Dexamethason in µg/L.

Parameter	EC_{50}	EC_{10}	NOEC	LOEC
Gesamtanzahl der Embryonen	n.d.	n.d.	≥ 105	> 105
Anzahl unbeschalter Embryonen	n.d.	n.d.	≥ 105	> 105
Anzahl beschalter Embryonen	n.d.	n.d.	< 1.06	≤ 1.06
Überleben	n.d.	n.d.	≥ 105	> 105
Wachstum	n.d.	n.d.	≥ 105	> 105

Sub caption of table – for example source, additional information.

Schlussfolgerung

Der Einfluss des Gestagens Dienogest und des Glucocortikoids Dexamethason auf die Reproduktionsleistung der Wasserschnecke *Potamopyrgus antipodarum* wurde jeweils in einem 28-tägigem Reproduktionstest nach OECD 242 untersucht.

Im Reproduktionstest mit Dienogest wurden die Schnecken geometrisch gemittelten, gemessenen Konzentrationen von 6.97, 11.2, 25.6, 106 und 332 ng/L Dienogest ausgesetzt. Nach 28 Tagen konnte kein signifikanter Unterschied zwischen den Kontrollen und den Behandlungsstufen in Bezug auf die untersuchten Parameter Reproduktion, Überleben und Wachstum festgestellt werden. Daher wurde die NOEC für jeden der Endpunkte auf ≥ 332 ng/L festgelegt.

Das Gestagen Dienogest zeigt somit bis zu einer Konzentration von 332 ng/L keine reproduktionstoxische Wirkung auf die Wasserschnecke *Potamopyrgus antipodarum*.

Im Reproduktionstest mit Dexamethason wurden die Schnecken geometrisch gemittelten, gemessenen Konzentrationen von 1.06, 3.54, 10.5, 36.3 und 105 µg/L Dexamethason ausgesetzt. Nach 28 Tagen konnte kein signifikanter Unterschied zwischen den Kontrollen und den Behandlungsstufen in Bezug auf die untersuchten Parameter Reproduktion (Gesamtanzahl der Embryonen und unbeschalte Embryonen), Überleben und Wachstum festgestellt werden. Daher wurde der NOEC für jeden der Endpunkte auf ≥ 105 µg/L festgelegt. Jedoch wurde eine

signifikante Abnahme der Anzahl an beschalten Embryonen in allen Behandlungsstufen beobachtet, was in einer NEOC von < 1.06 µg/L resultiert.

Gemäß der Richtlinie OECD 242 ist der relevante Parameter für die Berechnung der Konzentrationen des Reproduktionseffekts die Gesamtzahl der Embryonen, die hier keine substanzabhängige Wirkung zeigte. Daher wurde für die durchgeführte Studie für den Endpunkt Reproduktion eine NOEC von ≥ 105 µg/L abgeleitet. Das Glucocortikoid Dexamethason zeigt somit bis zu einer Konzentration von 105 µg/L keine reproduktionstoxische Wirkung auf die Wasserschnecke *Potamopyrgus antipodarum*.

1 Background and Aim of the Project

After more than two decades of intensive research, it has become clear that both humans and the environment - in particular the aquatic environment - are increasingly exposed to the influences of hormone-like substances. While in human populations this is reflected in increasing number of hormonal diseases, it leads to for examples growth disturbance or reduced reproductive performance of organisms in the environment. Many of the observed effects are attributed to chemicals known as endocrine disruptors (EDs). EDs are hormone-active substances that can cause changes in the hormonal system even in minor concentrations. The official definition of an ED was established during an international symposium in Weybridge (UK) dealing with endocrine disruptors: 'An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations' (WHO/IPCS 2002; Damstra, Bergman, Kavlock, & Van der Kraak, 2002).

The findings on specific effects of endocrine disruptors on aquatic organisms are described in the scientific literature. However, these findings are mostly related to fish, so that in the future the focus on other species such as aquatic invertebrates should be increased. In the scientific literature, there are indications that water snails react sensitively to hormone pollution in waters. Studies have shown that artificial hormones such as the estrogen receptor agonist 17α-ethinylestradiol has a reproductive effect in the New Zealand mud snail *Potamopyrgus antipodarum*. However, there are barley any findings on the effects of hormones on water snails in the regulatory context. In the development of tailored risk assessment concepts for endocrine active pharmaceutical substances (EAS), aquatic non-target organisms such as the water snails are to be looked at more closely in the future. This way, besides the consideration of fish, other equally sensitive groups of organisms will also be considered in the regulatory assessment.

In 2016, an OECD guideline (OECD TG 242) with *Potamopyrgus antipodarum*, which proved to be particularly sensitive to endocrine disruptors, was developed to investigate endocrine and reproductive toxic effects on water snails (7). In the chronic snail test, female snails are exposed to a test substance for 28 days. Mortality and reproduction of the animals are evaluated as endpoints. To measure the reproductive performance of the snails, the number of embryos in the brood pouch of each female is assessed at the end of the study.

The aim of the study is to gain new insights into the effects of progestins and glucocorticoids on water snails. Two medicinal substances for human use were tested: Dienogest [CAS No. 65928-58-7] as progestin and Dexamethasone [CAS No. 50-02-2] as artificial glucocorticoid. The results will be used to establish a tailored evaluation concept for environmental risks for progestins and glucocorticoids.

Before the start of the first snail reproduction test a Kick-Off-Meeting was held as a telephone conference on June 13, 2019. During this meeting the test design and the planned timeline were outlined. In addition, the extend of the analytical work was discussed.

This report summarizes the results of the primary performed literature research on the progestin Dienogest [CAS No. 65928-58-7] and the artificial glucocorticoid Dexamethasone [CAS No. 50-02-2]. In the following the results of the two performed studies with *Potamopyrgus antipodarum* exposed to Dienogest (1st study) and Dexamethasone (2nd study) are presented. The studies were performed according to OECD Guideline 242 (5) over 28 days under semi-static test conditions.

2 Work Package I: Planning and Preparation

2.1 Literature Research on Dienogest and Dexamethasone

Before starting the test, a literature research was performed on effects of progestins and glucocorticoids on molluscs, particular on snails. In addition, literature on other invertebrates were also considered. This work was part of the work package I described in the offer.

For the research, the preference was mainly on articles published in professional journals that were subjected to a peer review process. To ensure the quality of the selected literature, it was made sure that the results were discussed critically and, in addition, a comprehensible presentation of the methodology was given.

For the literature research, the following databases were used:

PubMed:

http://www.ncbi.nlm.nih.gov/pubmed

Science Direct

http://www.sciencedirect.com/

Web of Science:

http://apps.webofknowledge.com/UA_GeneralSearch_input.do?product=UA&search_mode=GeneralSearch&SID=S2lRkUdwKdY8zSZuMxd&preferencesSaved=

Google Scholar

https://scholar.google.de/

2.1.1 Dienogest

During the literature research for the progestin Dienogest, the search period was restricted to the last 6 years. Different terms including the following key words were searched:

- Progestins
- Progesterone
- Molluscs
- Snails
- Gastropods
- Gastropoda

In total, 13 reference were found of which four references were mainly dealing with effects of Cyproteron acetate (CPA), Levonorgestrel and progesterone on different snails and other invertebrate species. These references were selected to determine an appropriate concentration range for the snail test. In Table 5 the relevant results of the literature research are presented.

Table 5: Summary of the results of the literature research on Dienogest.

Test substance	Test organism	Test duration	Observed Endpoint	Effective concentrations	Ref.
Cyproteron actetate (CPA)	Lymnaea stagnalis	21 day exposure	Decreased reproduction (egg number)	2.0 µg/L	(1)
Cyproteron actetate (CPA)	Lymnaea stagnalis	21 day exposure	Increased frequency of polyembryony;	2.5 µg/L	(2)
			Decreased reproduction (egg number)	3.9 µg/L	
Levonorgestrel	Gammarus locusta	21 day exposure	Growth rates	10 ng/L	(3)
Progesterone	Marine mussel Mytilus galloprovinciales	7 day exposure	Gamete maturation and release	10 µg/L	(4)

Based on the results of the literature research and based on concentrations used in a fish study (ZEOGRT) with Dienogest (FKZ 3717 57 405 0) the concentration range was determined in agreement with the sponsor of the study. Since the main goal of the study was to clarify whether the snail *Potamopyrgus antipodarum* is less sensitive against the progestin Dienogest compared to zebrafish *Danio rerio*, which was used in the fish study, it was decided to use the following concentration range in the snail reproduction test:

3.20, 10.0, 32.0, 100 and 320 ng/L.

2.1.2 Dexamethason

During the literature research for the glucocorticoid Dexamethasone, the search period was restricted to the last 5 years. Different terms including the following key words were searched:

- Glucocorticoid
- Dexamethasone
- Corticosteroid
- Molluscs
- Snails
- Gastropods
- Gastropoda

No relevant references could be found describing effects of the glucocorticoid Dexamethasone on molluscs. Therefore, the concentration range for the snail reproduction test was determined based on the concentrations which were selected for the fish test (ZEOGRT) with Dexamethasone (FKZ 3717 57 405 0).

The following concentration range was used in the snail reproduction test with Dexamethasone:

1.0, 3.2, 10.0, 32.0 and 100 µg/L.

2.2 Health and Fecundity of *Potamopyrgus antipodarum* lab culture

The test organisms used for the test were bred in the laboratories of Fraunhofer since November 2017. Before the start of the test, the fecundity of the snails from the lab culture was proved to ensure that only healthy snails were introduced in the test. For this, tewnty randomly selected snails from the lab culture were selected and evaluated for their ability to reproduce. For this, their shell were cracked and embryos in the brood pouch under the shell were counted. According to the OECD Guideline 242, the mean number of embryos per snail to be used for the test should be between 5 and 20. These numbers reflect the lower and upper limit of mean embryo numbers in long-range laboratory cultures of *P. antipodarum*. The shell length of snails should be between 3.5 and 4.5 mm.

Total mean number of embryos before first test with Dienogest was 8.85 embryos with a mean length of 3.60 mm. Mean number of shelled embryos was 4.75 and mean number of unshelled embryos was 4.10.

Total mean number of embryos before first test with Dexamethasone was 12.4 embryos with a mean length of 3.67 mm. Mean number of shelled embryos was 5.35 and mean number of unshelled embryos was 7.05.

3 Work Package II & III: *Potamopyrgus antipodarum*, Reproduction Test according to OECD 242

3.1 Material and Methods

3.1.1 Test organisms

The species to be used in the test was the New Zealand mudsnail *Potamopyrgus antipodarum*. This freshwater mudsnail belongs to the phylum Mollusca, class Gastropoda, order Neotaenioglossa and family Hydrbiidae. Origin of the stock snails is the "Forschungsinstitut für Ökosystemanalyse und –bewertung e.V., Gaiac (Aachen, Germany)". Snails were bred in the laboratory of the Fraunhofer IME since November 2017.

Reproduction of *P. antipodarum* occurs throughout the whole year. The snails reproduce ovoviviparous. The eggs develop in a brood pouch under the shell of the snails. Older embryos are situated in the anterior and younger embryos in the posterior part of the brood pouch. When the development of the embryos has progressed far enough the egg shell tears up and they are released through the female aperture.

3.1.2 Test medium

Reconstituted (synthetic) water was used as test medium. The reconstituted water was prepared by mixing salt solutions of Tropic Marin® sea salt and sodium hydrogen carbonate ($NaHCO_3$) with deionized water. For preparation of the salt solutions at first, salt stock solutions were prepared. For this 75 g Tropic Marin® sea salt and 45.0 g $NaHCO_3$ were weighed, transferred to 1.0 L of deionized water and stirred for 24 hours. After stirring, 4 mL of each salt solution was distributed to 1000 mL deionized water, resulting in final salt concentrations of 0.3 g/L Tropic Marine sea salt® and 0.2 g/L $NaHCO_3$. The test medium was prepared in a container of inert material (glass) and aerated for about 24 hours before use.

The reconstituted water showed pH values of 8.0 ± 1.0, oxygen saturation > 60 %, conductivity of 770 ± 100 µS/cm and total organic carbon concentrations of < 2 mg/L.

3.1.3 Test containers

The test was performed with six replicates per test concentration with six snails per replicate under semi-static conditions with medium renewal three times per week. 600 mL glass beakers were used as test vessels and were filled with about 400 mL of test solution each. In each test vessel, an insert made of stainless-steel gauze with a mesh size of about 1 mm to enable unrestricted flow of the test medium, was inserted in each test vessel. The inserts were 15 cm high and had a diameter of 4.5 cm. The approximate volume of test solution enclosed by the inserts was about 150 mL. Each test vessel was aerated using a glass rod. A photograph of a test vessel used in the tests is presented in Figure 1. In addition, test vessels were covered to prevent snails from escaping.

Figure 1: Test container used in the tests with Dienogest and Dexamethasone.

Source: Fraunhofer IME

3.1.4 Food

Feeding was done daily with finely ground TetraPhyll® flakes (70 µg per animal per day). For application of the food, a homogenised suspension with deionised water was prepared. The appropriate amount of the suspension was pipetted in each test vessel once per day. During renewal of test solutions, any food remainings in the insert were removed when observed. The volume of suspension added to each test vessel was minimized to avoid a dilution of the test concentrations. The suspension was prepared immediately before use or stored freezed until use.

3.1.5 Replicates

Five concentrations with six replicates of six snails each (36 snails/concentration) were tested. In addition, control vessels without added test item were included in the test. Six replicates with six individuals per replicate were introduced as control.

3.1.6 Test concentrations and preparation of test solutions

Progestin Dienogest

The first study was carried out with the progestin Dienogest. The test substance was bought by Fraunhofer (Absource Diagnostics GmbH, CAS No. 65928-58-7). The following nominal test concentrations with a spacing factor of 3.16 were applied in the test:

3.20, 10.0, 32.0, 100, and 320 ng/L Dienogest.

For preparation of test solutions at first, for each test concentration a separate acetone application solution was prepared. For this, an appropriate amount of the test item was weighed and transferred to acetone. This stock solution was then diluted with acetone to prepare the application solutions for each treatment level.

For preparation of test solutions, a defined volume of the respective acetone application solution was pipetted on the glass wall of a glass bottle. For about one minute the empty glass bottles were aerated with synthetic air to remove the evaporated acetone from the bottles. After complete evaporation of acetone, the glass bottles were filled with 3 L of test medium. This procedure enables a better solubility of the test item in the water phase. Since acetone was

completely evaporated before adding of test medium, no solvent control was necessary. The test media were stirred for 24 hours and afterwards distributed to the replicates. An additional glass bottle which served for the control was introduced without any test item. The control was treated in the same way as the treatment levels. The test solutions were freshly prepared each day prior medium renewal.

Glucocorticoid Dexamethasone

The second study was performed with the artificial glucocorticoid Dexamethasone. The test substance was bought by Fraunhofer (abcr GmbH, CAS No. 50-02-2). The following nominal test concentrations with a spacing factor of 3.16 were applied in the test:

1.00, 3.20, 10.0, 32.0 and 100 µg/L Dexamethasone.

For preparation of test solutions at first, for each test concentration a separate application solution in methanol was prepared. For this, an appropriate amount of the test item was weighed and transferred to methanol. This stock solution was then diluted with methanol to prepare the application solutions for each treatment level.

For preparation of test solutions, a defined volume of the respective methanol application solution was pipetted on the glass wall of a glass bottle. For about one minute the empty glass bottles were aerated with synthetic air to remove the evaporated acetone from the bottles. After complete evaporation of methanol, the glass bottles were filled with 3 L of test medium. This procedure enables a better solubility of the test item in the water phase. Since methanol was completely evaporated before adding of test medium, no solvent control was necessary. The test media were stirred for 24 hours and afterwards distributed to the replicates. An additional glass bottle which served for the control was introduced without any test item. The control was treated in the same way as the treatment levels. The test solutions were freshly prepared each day prior medium renewal.

3.1.7 Test course

Six adult snails (3.5 – 4.5 mm shell length) per replicate (total 36 snails/concentration) were exposed to five nominal concentrations of the test item. In addition, a control without added test item was introduced. To begin the test, the test solutions were distributed to the test beakers and afterwards six snails per replicate were allocated randomly inside the insert placed in each test vessel containing the exposure medium using tweezers. Treatments were allocated to the test chamber and all subsequent handling of the test vessels was done in a random fashion in order to minimise any bias due to the position on the testing area. Between treatments and controls, a spatial separation was introduced to ensure that no test substance could spray across.

Snails were exposed in aerated test vessels under semi-static conditions with renewal of test media three times per week for 28 days. For test medium exchange, fresh test solution was distributed to six fresh (clean) replicate beakers per test concentration and controls, and temperature equilibrated. Then the inserts containing the snails were removed from the aged test solutions, drained, and placed in the freshly prepared beakers. Feeding was done daily with finely ground TetraPhyll® (70 µg per animal per day). The photoperiod was 16/8 hours throughout the test. Light intensity was 500 ± 100 lx at the surface of the test medium. The temperature of the test media was 16 ± 1.5 °C throughout the test. Water was aerated using glass pipettes connected to an air tubing system. The dissolved oxygen content was > 60%. The test vessels were aerated gently to avoid stripping of test item.

To avoid microbial growth, the inserts placed in each test vessel were replaced by clean inserts once per week.

Water quality parameters such as pH, oxygen saturation, conductivity and temperature, were measured alternating in one replicate per exposure group and control at each media renewal. Measurements of nitrate, ammonium and total nitrite content were performed alternating once per week in one replicate per concentration.

The test was performed in a temperature-controlled laboratory to ensure that the reconstituted water, which was applied to the aquaria, remained in the given range of 16 ± 1.5°C.

3.1.8 Observation and biological measurements

Test vessels were observed daily to achieve visual assessment of any abnormal behaviour (e.g. avoidance of water, avoidance of food, or lethargy, i.e. immobile snails fully retracted into the shell or detached from insert surface with foot protruded out of the shell). Any of these signs of stress were recorded if observed. If snails were found outside of control and test medium, they were transferred back to the medium immediately.

Mortality was recorded daily. Dead snails were removed from the test vessels. An animal was considered as dead when it was immobile, i.e. when it did not show any reaction after gently touching the foot or the operculum (in case of snails retracted into the shell) with a pair of tweezers.

The number of embryos in the brood pouch was analysed in all surviving snails at the end of the test. For this, snails were quick-frozen (in liquid nitrogen) and stored at -20 °C until analysis. The shell of the snails was then cracked carefully with a pair of pincers. Subsequently, the snails were placed into a dissecting dish containing a small volume of test medium or tap water. The soft body was prepared by removing the shell with dissecting needles or pointed tweezers. After removal of the shell, *P. antipodarum* embryos could easily be seen through the epithelia. The brood pouch of the snails was opened carefully with a dissecting needle. Subsequently, the embryos were removed and counted for determination of the reproductive success of each female. During counting, it was differentiated if the embryos were shelled or unshelled. If snails did not produce embryos, animals were sexed. Males are characterised by a penis in the neck (bottom of the mantle cavity behind the snout and the two ocular tentacles). Since no males occurred during the test, no further analysis regarding sex was performed.

Shell length of snails (from the tip of the shell to the lower edge of the aperture) was also determined at test end. The length, as a supporting parameter since shell length is positively correlated with female fecundity in *P. antipodarum*, was measured under a stereomicroscope with an ocular micrometre.

3.1.9 Chemical analysis

During the test, the concentration of the test item in the water phase was assessed by chemical analysis. Samples of freshly prepared test solutions were taken from the preparations before distributing it to the replicates. At media renewal, aged test solutions were sampled from the same representative test vessel per concentration. Samples of aged test solutions were taken from inside the insert. Sampling was performed in each treatment at test start and test end. In between only the lowest and highest treatment was analysed once weekly.

3.1.10 Evaluations and statistics

For each endpoint, the NOEC, LOEC, and, if possible, the EC_{50} and EC_{10} was determined. NOEC and LOEC were calculated by using ANOVA followed by multiple comparisons (e.g. Dunnett's test), or step-down trend tests (e.g. Williams' test) or an appropriate non-parametric test. As non-parametric alternatives, e.g., the Bonferroni-U-test according to Holm or the Jonckheere-Terpstra trend test could be used. Tests applied met the assumptions of the respective test (e.g. parametric tests met assumptions of distribution and homoscedasticity; trend tests were applied where monotonic dose response relationships were observed). When the test results showed a monotonic concentration-response relationship, the data was analysed by regression to determine the EC_X including the 95% confidence interval using Probit-analysis assuming log-normal distribution. To compute the EC_X, the complete data set was subjected to regression analysis. The computer software ToxRat® (6) was used for statistical evaluations. The appropriateness of ToxRat's automated test/model selection routines was assessed by expert judgement, and model selection decisions corrected, where necessary. Prior to reporting EC_X values, the model fit and the span of the 95% confidence intervals were evaluated.

The following endpoints observed in the reproduction test were evaluated quantitatively:

- Mortality (number of adults that were immobile following stimulation) on day 28
- Numbers of embryos (total, shelled and unshelled) in the brood pouch at the end of 28-day exposure period
- Individual lengths of adults
- Abnormal behaviour throughout the test
- Observation of morphological changes (at the end)

The purpose of this test was to determine the effect of the test substances Dienogest and Dexamethasone on the reproductive output of *P. antipodarum*. The survival of the parent animals and the embryo numbers in the brood pouch at the end of a 28-day of exposure period are also reported. For statistical evaluation of the embryo numbers, the mean number across replicates for each concentration was calculated.

For Dienogest, the deviation from the nominal concentration was greater than ± 20 % but concentrations has been satisfactorily maintained within ± 20% of the measured initial concentrations throughout the test. The evaluation of the concentration-effect-relationships and the calculations of effect concentrations was performed based on the geometric mean measured concentrations.

For Dexamethasone, the deviation from the nominal concentration was within ± 20 % and concentrations has been satisfactorily maintained within ± 20% of the measured initial concentrations throughout the test. The evaluation of the concentration-effect-relationships and the calculations of effect concentrations was based on the geometric mean measured concentrations.

3.2 Validity of the test

Both tests fulfil all validity criteria of the OECD guideline 242 (5) as:

- the mean mortality (accounting for all control replicates) did not exceed 20 % at the test end
- the mean number of embryos in the controls was at least 5 embryos per female at the test end
- the dissolved oxygen content was at least 60 % of the air saturation value in both control and exposure groups throughout the test; and
- mean temperature of test media was 16 ± 1.5 °C throughout the test in both control and exposure groups.

3.3 Results

3.3.1 Reproduction Test with Dienogest

3.3.1.1 Environmental conditions

Temperature during the test ranged from 16.5–17.6 °C (see Appendix A.1.3). The permitted temperature range of 16 ± 1.5 °C was exceeded by 0.1 °C on day 16 in concentrations of 6.97, 11.2, 25.6, 106 and 332 ng/L and on day 21 in the control and concentrations of 6.97, 25.6 and 106 ng/L.. However, this small increase is considered to be without influence on the outcome of the study. The dissolved oxygen concentration was between 7.60 mg/L and 9.00 mg/L, equivalent to 91.0 – 102.0 % oxygen saturation (see Appendix A.1.2). The pH values throughout the test were within a range of 7.49 – 8.36 in all treatment levels, the overall mean pH value was 8.10 (see Appendix A.1.2). Conductivity was between 698 and 768 µS/cm throughout the test (see Appendix A.1.2). Thus, all water quality criteria mentioned in the guideline (5) were met. The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in Lux. Light intensity was between 528 and 597 lx during the exposure period (see Appendix A.1.3). Concentrations of nitrate, nitrite and ammonia were between 1.0 – 4.0 mg/L, 0.001 – 0.012 mg/L and 0.01 – 0.04 mg/L, respectively (see Appendix A.1.2).

3.3.1.2 Measured exposure concentrations

The concentrations of the test item in the test media were determined by measurements of Dienogest in the aqueous phase of all treatment levels. The LOQ was set to 1.5 ng/L.

During the 28-days exposure period, measured concentrations ranged from 73.1 to 228 % of nominal, whereas the lowest test concentration of 3.2 ng/L showed recovery rates of 208 – 228 % of nominal while the other test concentrations showed recovery rates between 73.1 and 115 % of nominal. Freshly prepared test solutions were between 78.8 – 115 % and aged test solutions were between 73.1 and 113 % of nominal concentrations. The deviation of the measured test item concentrations from the nominal test concentrations was greater than 20%. However, concentrations remained stable within ± 20 % of measured initial concentrations (85 – 105 % of initial). The results were based on the geometric mean measured test item concentrations which were calculated based on the measured concentrations at test start (fresh and aged) and test end (fresh and aged). Only measured concentrations of test start and test end were used for calculations of geometric mean measured concentrations since only at tetst start

and test end all treatment levels were sampled. Geometric mean measured concentrations were 6.97, 11.2, 25.6, 106 and 332 ng/L (218, 112, 80.1, 106 and 104 % of nominal concentrations).

Measured test concentrations and geometric mean measured test concentrations used for evaluation are shown in Table 6 and Table 7.

Table 6: Measured Dienogest concentrations during the 28-day exposure.

	Measured concentrations [ng/L]									
Nominal Conc. [ng/L]	Day 0 fresh	Day 2 aged	Day 7 fresh	Day 9 aged	Day 14 fresh	Day 16 aged	Day 21 fresh	Day 23 aged	Day 26 fresh	Day 28 aged
Control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3.2	6.96	6.95	6.75	7.08	6.75	6.59	6.96	5.89	7.31	6.66
10.0	10.8	11.2	-	-	-	-	-	-	11.5	11.2
32.0	25.2	25.4	-	-	-	-	-	-	28.7	23.4
100	108	107	-	-	-	-	-	-	107	101
320	334	333	340	344	332	322	304	299	335	328

LOQ = 1.5 ng/L

Table 7: Geometric mean measured Dienogest concentrations during the 28-day exposure used for the effect assessment.

Nominal Concentration [ng/L]	Geometric mean measured Concentration [ng/L]	Geometric mean measured Concentration [% of nominal]
Control	<LOQ	-
3.2	6.97	218
10.0	11.2	112
32.0	25.6	80.1
100	106	106
320	332	104

LOQ = 1.5 ng/L

3.3.1.3 Survival, Growth, physical/pathological symptoms and changes in behaviour

No snail died in the control or any treatment. Thus, no concentration related mortality of the test organisms was observed.

In addition, no significant difference in the body length of individuals was shown between control and any test concentration. Mean length of control snails was 3.61 mm and mean length of snails in treatments ranged from 3.58 to 3.61 mm.

Results of mortality and growth are shown in Table 8 and Table 9

The NOEC for both survival and growth were identified to be ≥ 332 ng/L.

The test organisms showed no abnormalities in their behaviour (e.g. avoidance of water or lethargy). No other clinical signs were observed in any replicate at any concentration tested. Neither any physical nor pathological symptoms were seen.

Table 8: Survival data and percent reduction of survival after 28-day exposure to Dienogest (n=36).

Geometric mean measured concentration [ng/L]	Parental survival	Reduction of survival [%] (Significance)
Control	36	0
6.97	36	0 (-)
11.2	36	0 (-)
25.6	36	0 (-)
106	36	0 (-)
332	36	0 (-)

(-) no statistically significant difference between control and treatments

Step-down Cochran-Armitage Test, significance was alpha = 0.05, one sided greater

Table 9: Growth data and percent reduction of length after 28-day exposure to Dienogest.

Geometric mean measured concentration [ng/L]	Length on day 28 Mean ± SD [mm]	Decrease in length [%] (Significance)
Control	3.61 ± 0.046	-
6.97	3.59 ± 0.049	0.416 (-)
11.2	3.61 ± 0.033	-0.092 (-)
25.6	3.58 ± 0.046	0.878 (-)
106	3.61 ± 0.023	-0.092 (-)
332	3.60 ± 0.044	0.092 (-)

(-) no statistically significant difference between control and treatments

Dunnett's test, significance was alpha = 0.05, one sided smaller

3.3.1.4 Reproduction

During evaluation of reproduction results, embryo numbers were differentiated in total, shelled and unshelled embryos. The total number of embryos ranged from 7.18 to 9.32 across all treatments and controls. The number of shelled embryos and unshelled embryos ranged from 3.58 to 4.43 and 3.65 to 4.85 embryos, respectively.

There was no statistically significant effect on the number of total, shelled and unshelled embryos compared to the control.

Table 10 and Figure 2 show the results of the reproduction at the end of the 28-day exposure period related to the total number of embryos.

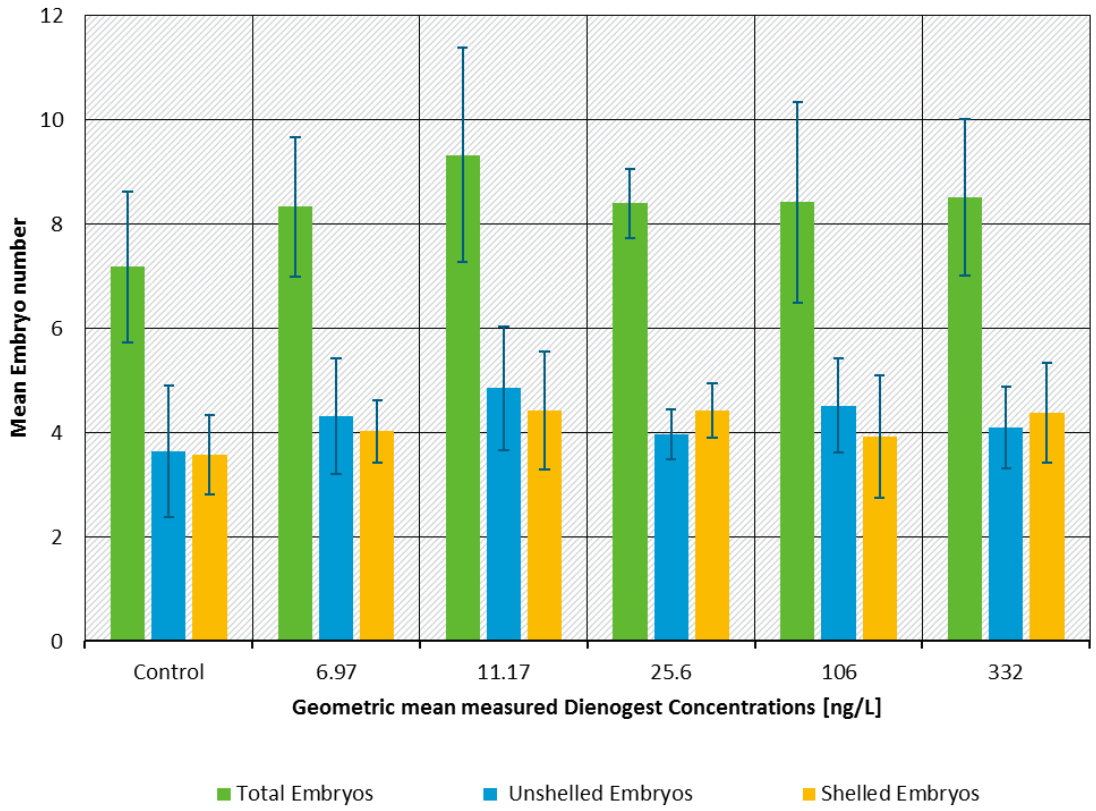
Table 10: Reproduction data of total embryos after 28-day exposure to Dienogest.

Geometric mean measured concentration [ng/L]	Mean number of total embryos per replicate [Mean ± SD]	% Reproduction Decrease (Significance)
Control	7.18 ± 1.44	
6.97	8.33 ± 1.33	16.0 (-)
11.2	9.32 ± 2.06	29.7 (-)
25.6	8.40 ± 0.67	16.9 (-)
106	8.42 ± 1.92	17.2 (-)
332	8.52 ± 1.50	18.6 (-)

(-) no statistically significant difference between control and treatments

Dunnett's test, significance was alpha = 0.05, one sided smaller

Figure 2: Embryo numbers (total, shelled and unshelled) at the end of the 28-day exposure to Dienogest.



Error bars demonstrate the standard deviation over the mean number of embryos. reference: Fraunhofer IME

3.3.1.5 Effect and No Observed Effect Concentrations

Since no mortality was observed in any treatment, no probit anaylsis was performed for this endpoint. The NOEC was set to be ≥ 332 ng/L.

No statistically significant concentration-response was found for the length of snails at test end, thus, no effect concentrations could be calculated for growth. The NOEC was determined to be ≥ 332 ng/L.

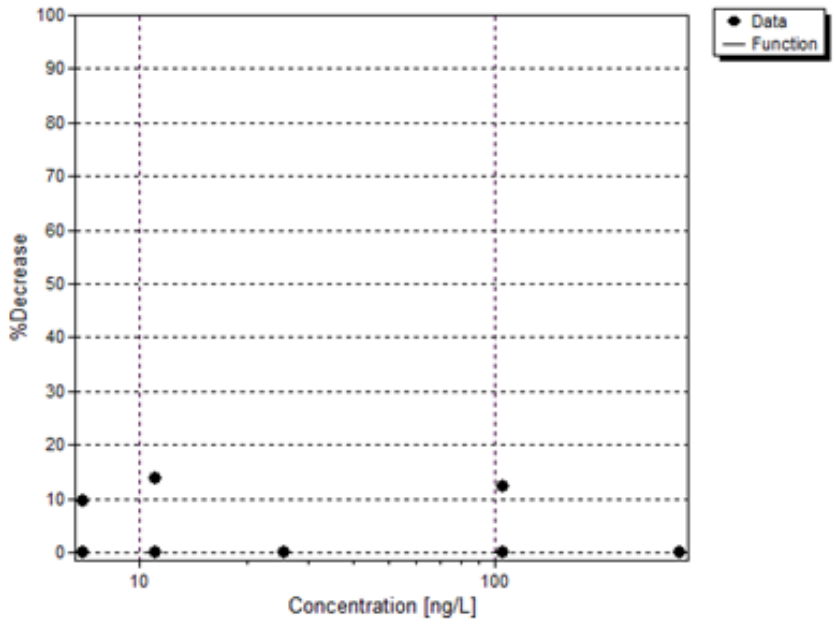
For the assessment of effects on embryo numbers and for calculation of effect concentrations the mean numbers of embryos per replicate were used. For calculations, embryo numbers were differentiated in total, shelled and unshelled embryos. The reproduction of *P. antipodarum* after 28 days related to the total number of embryos is presented in Figure 3 (generated during probit analysis with ToxRat® (6)).

By using probit analysis, no statistically significant concentration effect relationship could be determined neither for total, shelled or unshelled embryo numbers. Thus, no effect concentrations (EC_{10}, EC_{20} and EC_{50}) were calculated.

For calculations of NOEC and LOEC, hypothesis testing in order to determine statistically significant differences was performed by comparing treatments against the control using Dunnett's t-test (significance level alpha = 0.05, one sided smaller).

Since no significant difference in the reproduction between embryo numbers (total, shelled and unshelled embryo numbers) and controls were observed, the NOEC for reproduction was determined to be ≥ 332 ng/L.

Figure 3: Decrease (%) of total embryo numbers compared to the control showing the influence of the test item Dienogest on reproduction of the introduced *P. antipodarum* as observed under presence of the test item after 28 days.



Source: ToxRat® Professional version 3.3.0 (6).

3.3.1.6 Conclusion

The 28-day toxicity of Dienogest on the test organism *Potamopyrgus antipodarum* was determined according to OECD 242 (5). Snails were exposed to geometric mean measured concentrations of 6.97, 11.2, 25.6, 106 and 332 ng/L.

After 28 days, no significant difference was observed between control and any treatment regarding the investigated parameters reproduction, growth and mortality. Therefore, the NOEC for each endpoint was set to be ≥ 332 ng/L.

The progestin Dienogest therefore has no toxic effect on reproduction of the water snail *Potamopyrgus antipodarum* up to a concentration of 332 ng/L.

3.3.2 Reproduction Test with Dexamethasone

3.3.2.1 Environmental conditions

Temperature during the test ranged from 16.4–17.5 °C (see Appendix A.2.3). The permitted temperature range of 16 ± 1.5 °C was maintained. The dissolved oxygen concentration was between 7.50 mg/L and 9.40 mg/L, equivalent to 88.0 – 105.0 % oxygen saturation (see Appendix A.2.2). The pH values throughout the test were within a range of 7.70 – 8.51 in all treatment levels, the overall mean pH value was 8.13 (see Appendix A.1.2). Conductivity was between 723 and 782 µS/cm throughout the test (see Appendix A.2.2). Thus, all water quality criteria mentioned in the guideline (5) were met. The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in Lux. Light intensity was between 524 and 588 lx during the exposure period (see Appendix A.2.3). Concentrations of nitrate, nitrite and ammonia were between 1.0 – 8.0 mg/L, 0.0 – 0.116 mg/L and 0.0 – 0.01 mg/L, respectively. DOC of test medium was measured once during the study and was at 1.014 – 1.017 mg/L (see Appendix A.2.2).

3.3.2.2 Measured exposure concentrations

The concentrations of the test item in the test media were determined by measurements of Dexamethasone in the aqueous phase of all treatment levels. The LOQ was set to 0.1 µg/L.

During the 28-day exposure period, measured concentrations ranged from 96.6 to 120 % of nominal. Freshly prepared test solutions were between 99.7 – 116 % and aged test solutions between 96.6 and 120 % of nominal concentrations. The deviation of the measured test item concentrations from the nominal test concentrations was within ± 20%. In addition, concentrations remained stable within ± 20 % of measured initial concentrations (91.0 – 117 % of initial). The results were based on the geometric mean measured test item concentrations which were calculated based on the measured concentrations at test start (fresh and aged) and test end (fresh and aged). Only measured concentrations of test start and test end were used for calculations of geometric mean measured concentrations since only at tetst start and test end all treatment levels were sampled. Geometric mean measured concentrations of 1.06, 3.54, 10.5, 36.3 and 105 µg/L (106, 111, 105, 114 and 105 % of nominal concentrations) were used for evaluation of effects.

Measured test concentrations and geometric mean measured test concentrations used for evaluation are shown in Table 11 and Table 12.

Table 11: Measured Dexamethasone concentrations during the 28-day exposure.

	Measured concentrations [µg/L]									
Nominal Conc. [µg/L]	Day 0 fresh	Day 2 aged	Day 7 fresh	Day 9 aged	Day 14 fresh	Day 16 aged	Day 21 fresh	Day 23 aged	Day 26 fresh	Day 28 aged
Control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1.00	0.997	1.04	1.02	1.20	1.03	1.17	1.12	1.15	1.11	1.08
3.20	3.42	3.48	-	-	-	-	-	-	3.69	3.60
10.0	10.6	10.6	-	-	-	-	-	-	10.3	10.6
32.0	36.3	34.3	-	-	-	-	-	-	37.2	37.6
100	112	102	109	107	104	103	105	96.6	105	101

LOQ = 0.1 µg/L

Table 12: Geometric mean measured Dexamethasone concentrations during the 28-day exposure used for the effect assessment.

Nominal Concentration [µg/L]	Geometric mean measured Concentrations [µg/L]	Geometric mean measured Concentrations [% of nominal]
Control	<LOQ*	-
1.00	1.06	106
3.20	3.54	111
10.0	10.5	105
32.0	36.3	114
100	105	105

LOQ = 0.1 µg/L

3.3.2.3 Survival, Growth, physical/pathological symptoms and changes in behaviour

No snail died in the control or any treatment. Thus, no concentration related mortality of the test organisms was observed.

In addition, no significant difference in the body length of individuals was shown between control and any test concentration. Mean length of control snails was 3.76 mm and mean length of snails in treatments ranged from 3.67 to 3.78 mm.

Results of mortality and growth are shown in Table 13 and Table 14.

The NOEC for both survival and growth were identified to be ≥ 105 µg/L.

The test organisms showed no abnormalities in their behaviour (e.g. avoidance of water or lethargy). No other clinical signs were observed in any replicate at any concentration tested. Neither any physical nor pathological symptoms were seen.

Table 13: Survival data and percent reduction of survival after 28-day exposure to Dexamethasone (n=36).

Geometric mean measured concentration [µg/L]	Parental survival	Reduction of survival [%] (Significance)
Control	36	0
1.06	36	0 (-)
3.54	36	0 (-)
10.5	36	0 (-)
36.3	36	0 (-)
105	36	0 (-)

(-) no statistically significant difference between control and treatments

Step-down Cochran-Armitage Test, significance was alpha = 0.05, one sided greater

Table 14: Growth data and percent reduction of length after 28-day exposure to Dexamethasone.

Geometric mean measured concentration [µg/L]	Length on day 28 Mean ± SD [mm]	Decrease in length [%] (Significance)
Control	3.76 ± 0.122	-
1.06	3.69 ± 0.033	1.77 (-)
3.54	3.67 ± 0.068	2.35 (-)
10.5	3.73 ± 0.072	0.843 (-)
36.3	3.78 ± 0.092	-0.488 (-)
105	3.69 ± 0.084	1.69 (-)

(-) no statistically significant difference between control and treatments

Dunnett's test, significance was alpha = 0.05, one sided smaller

3.3.2.4 Reproduction

During evaluation of reproduction results, embryo numbers were differentiated in total, shelled and unshelled embryos. The total number of embryos ranged from 12.4 to 15.8 across all treatments and controls. The number of shelled embryos and unshelled embryos ranged from 5.80 to 8.55 and 6.38 to 8.15 embryos, respectively.

For the total number of embryos, a statistically significant decrease was determined for the second treatment level of 3.54 µg/L. However, concentrations below and above 3.54 µg/L showed no statistically significant difference compared to the control.

For shelled embryos, a statistically significant decrease of embryo numbers was observed compared to the control for all treatment levels.

For unshelled embryos, no statistically significant decrease in the embryo number was observed. Contrarily, a statistically significant increase of unshelled embryos was observed for the treatment of 36.3 µg/L. However, treatment levels below and above 36.3 µg/L showed no significant increase in the number of unshelled embryos.

Table 15 and Figure 4 show the results of the reproduction at the end of the 28-days exposure period related to the total number of embryos.

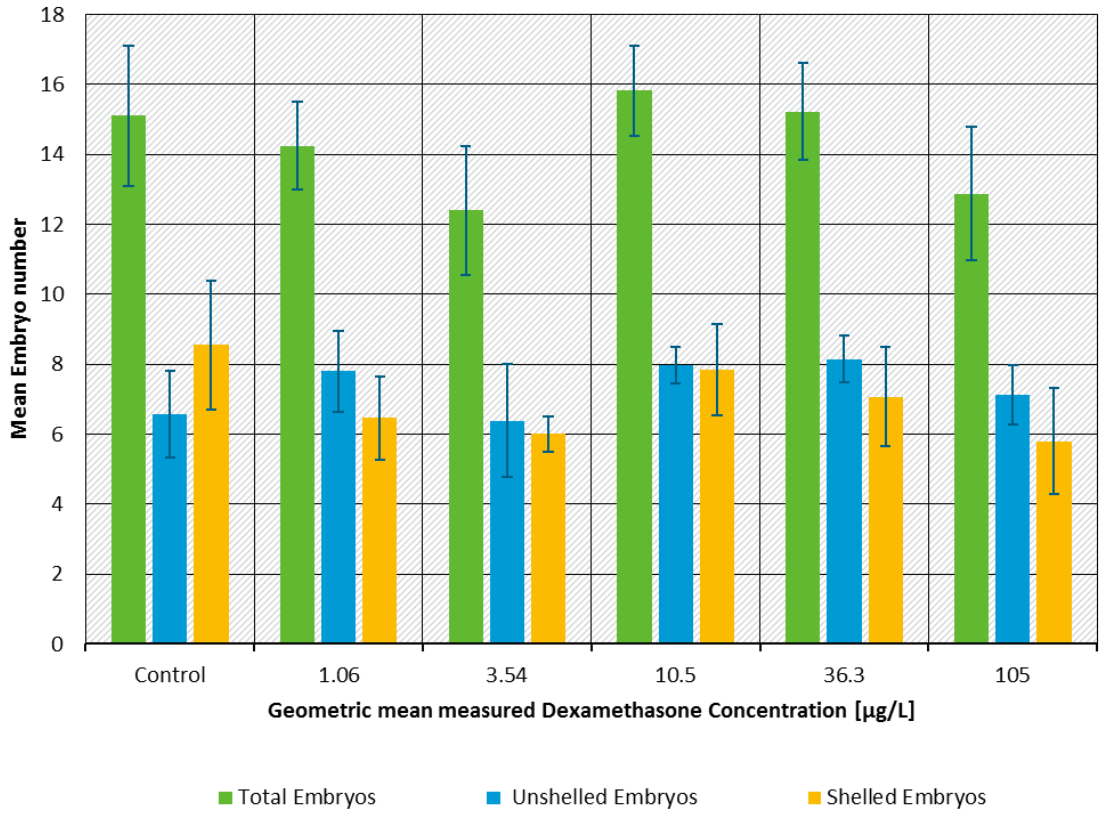
Table 15: Reproduction data of total embryos after 28-day exposure to Dexamethasone.

Geometric mean measured concentration [µg/L]	Mean number of total embryos per replicate [Mean ± SD]	% Reproduction Decrease (Significance)
Control	15.1 ± 2.02	
1.06	14.3 ± 1.25	5.63 (-)
3.54	12.4 ± 1.84	17.88 (+)
10.5	15.8 ± 1.29	-4.75 (-)
36.3	15.2 ± 1.39	-0.773 (-)
105	12.9 ± 1.91	14.7 (-)

(-) no statistically significant difference between control and treatments

Dunnett's test, significance was alpha = 0.05, one sided smaller

Figure 4: Embryo numbers (total, shelled and unshelled) at the end of the 28-day exposure to Dexamethasone.



Error bars demonstrate the standard deviation over the mean number of embryos. reference: Fraunhofer IME

3.3.2.5 Effect and No Observed Effect Concentrations

Since no mortality was observed in any treatment, no probit anaylsis was performed for this endpoint. The NOEC was set to be ≥ 105 µg/L.

No statistically significant concentration-response was found for the length of snails at test end and, thus, no effect concentrations could be calculated for growth. The NOEC was determined to be ≥ 105 µg/L.

For the assessment of effects on embryo numbers and for calculation of effect concentrations the mean numbers of embryos per replicate were used. For calculations, embryo numbers were differentiated in total, shelled and unshelled embryos. The reproduction of *P. antipodarum* after 28 days related to the total number of embryos is presented in Figure 5 (generated during probit analysis with ToxRat® (6)) .

By using probit analysis, no statistically significant concentration effect relationship could be determined neither for total, shelled or unshelled embryo numbers. Thus, no effect concentrations (EC_{10}, EC_{20} and EC_{50}) were calculated.

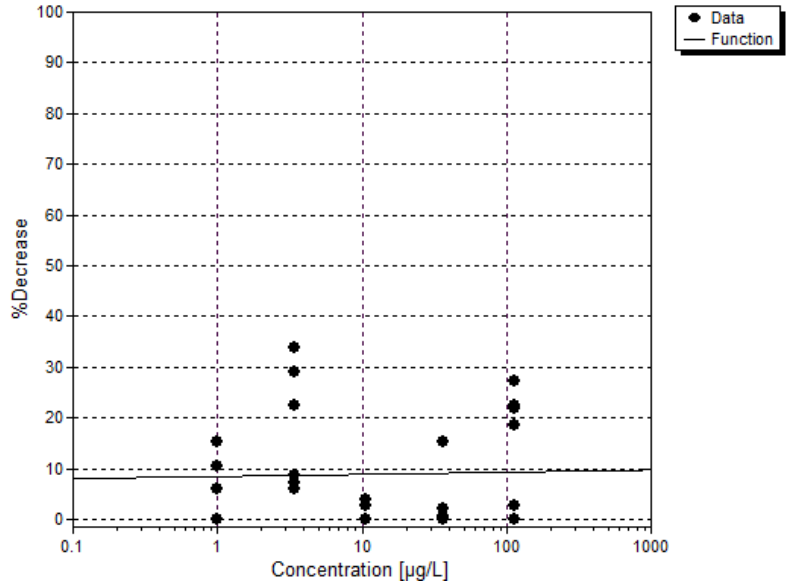
For calculations of NOEC and LOEC, hypothesis testing in order to determine statistically significant differences was performed by comparing treatments against the control using Dunnett´s Multiple t-test or Williams Multiple Sequential t-test procedures (significance level alpha = 0.05, one sided smaller).

For total embryo numbers, a significant decrease between embryos in the treatment of 3.54 µg/L and the control was observed. However, no significant effect occurred in treatments below and above 3.54 µg/L. Therefore, the NOEC for reproduction related to the total number of embryos was determined to be ≥ 105 ng/L.

For shelled embryos, a significant decrease in embryo numbers was observed for all treatment levels. Therefore, the NOEC for shelled embryos was determined to be < 1.06 µg/L.

For unshelled embryos, no significant decrease in the embryo number was observed. In contrast, a significant increase was observed between the treatment of 36.3 µg/L and the control (Dunnett's test, significance level alpha = 0.05, one sided greater). However, treatment levels below and above 36.3 µg/L showed no significant increase compared to the control. Thus, the NOEC was determined to be ≥ 105 µg/L.

Figure 5: Decrease (%) of total embryo numbers compared to the control showing the influence of the test item Dexamethasone on reproduction of the introduced *P. antipodarum* as observed under presence of the test item after 28 days.



Source: ToxRat® Professional version 3.3.0 (6).

3.3.2.6 Conclusion

The 28-day toxicity of Dexamethasone on the test organism *Potamopyrgus antipodarum* was determined according to OECD 242 (5). Snails were exposed to geometric mean measured concentrations of 1.06, 3.54, 10.5, 36.3 and 106 µg/L.

After 28 days, no significant difference was observed between control and any treatment regarding the investigated parameters reproduction (in terms of total embryos), growth and mortality. Therefore, the NOEC for each endpoint was set to be ≥ 105 µg/L.

In contrast, a significant decrease of embryo numbers in terms of shelled embryos was observed for all treatment levels, resulting in a NOEC of < 1.06 µg/L. For unshelled embryos, no significant difference between embryo numbers in any treatment compared to the control were observed, resulting in a NOEC of ≥ 105 µg/L

According to the guideline OECD 242, the relevant parameter for calculation of reproductive effect concentrations is the total number of embryos, which showed no test item related effect. Therefore, the NOEC derived for the performed study was ≥ 105 µg/L.

The glucocorticoid Dexamethasone therefore has no toxic effect on reproduction of the water snail *Potamopyrgus antipodarum* up to a concentration of 105 ng/L.

4 List of references

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(5) OECD Guideline for Testing of Chemicals, Sect. 2: Effects on Biotic Systems, N. 242: "*Potamopyrgus antipodarum*, Reproduction Test". Adopted July 2016

(6) ToxRat® Professional 3.3.1, ToxRat® Solutions GmbH, Naheweg 15, D-52477 Alsdorf (http://www.toxrat-solutions.de).

(7) Geiss C., Ruppert K., Oehlmann J. German Environment Agency, April 2017. Validation of reproduction tests with molluscs – Establishment of an OECD test guideline for the identification of endocrine reproductive toxic effects in snails

A Appendix

A.1 Processed raw data – Reproduction test with Dienogest

A.1.1 Fecundity of snails before test start

Table 16: Fecundity and Length of 20 randomly selected snails before test start (Dienogest).

Subtitle *[if not necessary, please remove]*

	Embryo number			
Snail No.	**Shelled**	**unshelled**	**total**	**Length**
1	1	5	6	3.74
2	4	6	10	3.61
3	3	9	12	3.51
4	7	6	13	3.53
5	4	0	4	3.69
6	7	6	13	3.50
7	5	4	9	3.63
8	2	5	7	3.53
9	5	3	8	3.63
10	6	1	7	3.51
11	8	5	13	3.74
12	6	6	12	3.77
13	4	1	5	3.51
14	4	6	10	3.61
15	3	1	4	3.50
16	10	7	17	3.81
17	6	0	6	3.74
18	4	0	4	3.50
19	2	5	7	3.51
20	4	6	10	3.50
Mean	**4.8**	**4.1**	**8.9**	**3.60**

Sub caption of table – for example source, additional information.

A.1.2 Water chemistry data

Table 17: Oxygen concentration of the overlying water [mg/L] during the test with Dienogest.

Concentrations given as measured geometric mean measured concentrations [ng/L].

Date	Control		6.97		11.2		25.6		106		332	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	-	8.50	-	8.70	-	8.50	-	8.60	-	8.70	-	8.50
Day 2	8.60	8.40	8.70	8.30	8.70	8.20	8.70	8.40	8.60	8.60	8.50	8.50
Day 5	8.90	8.80	8.93	8.64	8.79	8.63	8.91	8.72	8.90	8.66	8.97	8.73
Day 7	8.97	8.28	8.99	8.61	8.70	8.68	8.97	8.74	8.88	8.76	9.00	8.62
Day 9	8.80	8.70	8.70	8.60	8.80	8.60	8.70	8.60	8.70	8.50	8.80	8.50
Day 12	8.60	8.50	8.80	8.50	8.80	8.50	8.70	8.60	8.70	8.60	8.80	8.60
Day 14	8.50	8.40	8.70	8.30	8.70	8.30	8.80	8.20	8.70	8.50	8.70	8.60
Day 16	8.40	8.70	8.60	8.40	8.60	8.50	8.50	8.30	8.60	8.30	8.50	8.50
Day 19	8.50	7.60	8.40	7.90	8.40	8.10	8.40	7.90	8.40	8.10	8.50	8.50
Day 21	8.60	8.60	8.60	8.50	8.80	8.50	8.50	8.50	8.70	8.50	8.60	8.40
Day 23	8.60	8.50	8.60	8.40	8.60	8.30	8.80	8.40	8.80	8.60	8.60	8.60
Day 26	8.30	8.40	8.60	8.40	8.70	8.50	8.50	8.60	8.70	8.30	8.20	8.40
Day 28	8.30	-	8.20	-	8.30	-	8.30	-	8.20	-	8.20	-

Values of the parallel test vessels throughout the test.

Table 18: Oxygen saturation of the overlying water [%] during the test with Dienogest.

Concentrations given as geometric mean measured concentrations [ng/L].

Date	Control		6.97		11.2		25.6		106		332	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	-	96.0	-	96.0	-	99.0	-	99.0	-	99.0	-	96.0
Day 2	101.0	98.0	100.0	98.0	101.0	97.0	101.0	99.0	100.0	99.0	100.0	100.0
Day 5	98.6	98.7	97.6	96.4	97.1	95.4	98.5	96.7	98.0	96.0	98.2	96.7
Day 7	98.5	91.0	98.4	94.4	97.0	94.8	99.2	95.7	98.5	95.8	99.5	94.2
Day 9	100.0	102.0	97.0	97.0	100.0	100.0	98.0	97.0	100.0	100.0	100.0	97.0
Day 12	99.0	97.0	98.0	98.0	99.0	98.0	98.0	96.0	99.0	96.0	99.0	98.0
Day 14	97.0	97.0	99.0	98.0	100.0	97.0	101.0	96.0	100.0	96.0	100.0	98.0
Day 16	99.0	101.0	99.0	98.0	100.0	100.0	100.0	98.0	101.0	98.0	100.0	100.0
Day 19	98.0	94.0	99.0	95.0	98.0	95.0	96.0	93.0	97.0	96.0	97.0	97.0
Day 21	100.0	101.0	98.0	99.0	102.0	97.0	98.0	99.0	99.0	97.0	98.0	96.0
Day 23	97.0	96.0	96.0	96.0	100.0	97.0	100.0	97.0	100.0	100.0	98.0	97.0
Day 26	96.0	97.0	96.0	96.0	99.0	95.0	98.0	98.0	98.0	91.0	98.0	94.0
Day 28	96.0	-	95.0	-	96.0	-	93.0	-	94.0	-	94.0	-

Values of the parallel test vessels throughout the test.

Table 19: pH of the overlying water during the test with Dienogest.

Concentrations given as geometric mean measured concentrations [ng/L].

Date	Control		6.97		11.2		25.6		106		332	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	-	7.54	-	7.55	-	7.54	-	7.50	-	7.49	-	7.49
Day 2	8.07	8.19	8.07	8.16	8.07	8.16	8.06	8.11	8.07	8.10	8.06	8.09
Day 5	8.29	8.13	8.30	8.21	8.33	7.93	8.32	8.23	8.33	8.26	8.36	8.25
Day 7	8.29	8.21	8.29	8.23	8.29	8.23	8.28	8.25	8.31	8.26	8.28	8.27
Day 9	8.04	8.07	8.02	8.07	8.02	8.06	8.04	8.05	8.01	8.04	8.02	8.04
Day 12	8.11	8.17	8.12	8.13	8.11	8.13	8.09	8.15	8.12	8.09	8.09	8.09
Day 14	8.13	8.15	8.14	8.13	8.12	8.11	8.13	8.10	8.10	8.08	8.10	8.08
Day 16	8.13	8.10	8.15	8.10	8.13	8.11	8.13	8.11	8.14	8.09	8.15	8.06
Day 19	8.14	8.34	8.14	8.29	8.12	8.28	8.13	8.26	8.09	8.25	8.12	8.20
Day 21	8.21	8.21	8.22	8.20	8.21	8.19	8.19	8.19	8.20	8.18	8.20	8.16
Day 23	8.20	8.29	8.21	8.29	8.21	8.28	8.21	8.25	8.20	8.25	8.19	8.21
Day 26	8.13	8.20	8.11	8.20	8.14	8.21	8.14	8.19	8.10	8.17	8.13	8.14
Day 28	8.25	-	8.24	-	8.21	-	8.20	-	8.19	-	8.19	-

Values of the parallel test vessels throughout the test.

Table 20: Conductivity of the overlying water [µS/cm] during the test with Dienogest.

Concentrations given as geometric mean measured concentrations [ng/L].

Date	Control		6.97		11.2		25.6		106		332	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	-	719	-	720	-	718	-	717	-	718	-	717
Day 2	722	739	722	736	721	732	721	733	722	732	721	733
Day 5	738	746	739	746	736	746	737	745	736	745	737	745
Day 7	752	733	749	724	749	725	751	724	749	724	749	724
Day 9	730	747	729	740	729	739	728	739	730	739	728	738
Day 12	748	768	748	749	746	747	745	749	750	746	746	748
Day 14	755	736	754	735	751	733	752	733	752	732	753	734
Day 16	745	702	744	701	741	700	743	699	744	698	743	700
Day 19	708	734	705	729	705	732	707	733	705	738	705	732
Day 21	737	712	736	713	733	712	735	711	735	711	734	710
Day 23	721	727	719	727	716	726	715	726	717	727	715	726
Day 26	735	743	731	743	729	743	732	743	730	742	730	740
Day 28	744	-	743	-	744	-	744	-	745	-	744	-

Values of the parallel test vessels throughout the test.

Table 21: Weekly measurements of nitrate in the overlying water [mg/L] during the test with Dienogest.

Concentrations given as geometric mean measured concentrations [ng/L].

Date	Control		6.97		11.2		25.6		106		332	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	-	1.00	-	2.00	-	3.00	-	3.00	-	3.00	-	3.00
Day 2	2.00	-	2.00	-	2.00	-	2.00	-	1.00	-	2.00	-
Day 7	-	1.00	-	2.00	-	2.00	-	3.00	-	3.00	-	3.00
Day 9	2.00	-	3.00	-	3.00	-	2.00	-	2.00	-	4.00	-
Day 14	-	1.00	-	2.00	-	1.00	-	2.00	-	2.00	-	1.00
Day 16	1.00	-	1.00	-	1.00	-	1.00	-	2.00	-	1.00	-
Day 21	-	1.00	-	1.00	-	1.00	-	2.00	-	2.00	-	2.00
Day 23	2.00	-	2.00	-	2.00	-	2.00	-	1.00	-	1.00	-

Values of the parallel test vessels throughout the test.

Table 22: Weekly measurements of nitrite in the overlying water [mg/L] during the test with Dienogest.

Concentrations given as geometric mean measured concentrations [ng/L].

Date	Control		6.97		11.2		25.6		106		332	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	-	0.005	-	0.003	-	0.004	-	0.007	-	0.003	-	0.006
Day 2	0.007	-	0.012	-	0.009	-	0.009	-	0.006	-	0.005	-
Day 7	-	0.004	-	0.004	-	0.004	-	0.004	-	0.005	-	0.005
Day 9	0.001	-	0.001	-	0.001	-	0.001	-	0.001	-	0.001	-
Day 14	-	0.007	-	0.006	-	0.006	-	0.006	-	0.006	-	0.008
Day 16	0.002	-	0.002	-	0.003	-	0.002	-	0.002	-	0.002	-
Day 21	-	0.007	-	0.006	-	0.006	-	0.006	-	0.005	-	0.007
Day 23	0.003	-	0.001	-	0.002	-	0.002	-	0.002	-	0.001	-

Values of the parallel test vessels throughout the test.

Table 23: Weekly measurements of ammonia in the overlying water [mg/L] during the test with Dienogest.

Concentrations given as geometric mean measured concentrations [ng/L].

Date	Control		6.97		11.2		25.6		106		332	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	-	0.04	-	0.03	-	0.04	-	0.03	-	0.03	-	0.03
Day 2	0.01	-	0.02	-	0.02	-	0.01	-	0.01	-	0.01	-
Day 7	-	0.03	-	0.02	-	0.02	-	0.01	-	0.02	-	0.02
Day 9	0.01	-	0.00	-	0.00	-	0.00	-	0.00	-	0.01	-
Day 14	-	0.02	-	0.03	-	0.04	-	0.02	-	0.02	-	0.03
Day 16	0.01	-	0.01	-	0.02	-	0.01	-	0.01	-	0.01	-
Day 21	-	0.01	-	0.01	-	0.03	-	0.01	-	0.01	-	0.01
Day 23	0.01	-	0.01	-	0.02	-	0.02	-	0.01	-	0.01	-

Values of the parallel test vessels throughout the test.

A.1.3 Temperature and Light

Table 24: Temperature of the overlying water [°C] during the test with Dienogest.

Concentrations given as geometric mean measured concentrations [ng/L].

Date	Control		6.97		11.2		25.6		106		332	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	-	17.0	-	17.2	-	17.2	-	17.4	-	17.4	-	17.4
Day 2	17.0	17.0	17.0	17.3	17.0	17.5	17.0	17.5	17.0	17.4	17.0	17.4
Day 5	16.6	16.5	16.8	16.8	16.9	17.1	16.9	17.2	16.8	17.1	16.9	17.2
Day 7	16.9	16.9	17.0	17.3	17.0	17.3	17.0	17.4	17.0	17.4	17.0	17.4
Day 9	16.9	16.9	17.0	17.1	17.0	17.4	17.0	17.4	17.0	17.0	17.1	17.3
Day 12	17.1	17.5	17.2	17.5	17.1	17.5	17.2	17.5	17.2	17.5	17.2	17.5
Day 14	17.5	16.7	17.5	17.4	17.5	17.5	17.5	17.4	17.5	17.4	17.5	17.5
Day 16	17.5	17.3	17.6	17.5	17.6	17.5	17.6	17.5	17.5	17.5	17.6	17.5
Day 19	17.5	17.5	17.3	17.5	17.4	17.5	17.3	17.5	17.5	17.5	17.3	17.5
Day 21	17.6	17.5	17.6	17.5	17.5	17.5	17.6	17.5	17.6	17.5	17.5	17.5
Day 23	17.4	17.3	17.4	17.5	17.3	17.4	17.4	17.5	17.4	17.5	17.4	17.4
Day 26	17.0	17.2	17.1	17.3	17.1	17.3	17.1	17.4	17.1	17.3	17.2	17.4
Day 28	16.9	-	16.9	-	16.8	-	16.8	-	17.0	-	16.9	-

Table 25: Light intensity [lx] in the test chamber throughout the test during the test with Dieongest.

Date	Light intensity [lx
Day 0	576
Day 2	594
Day 5	593
Day 7	529
Day 9	528
Day 12	560
Day 14	579
Day 16	581
Day 19	585
Day 21	595
Day 23	597
Day 26	588
Day 28	590

A.1.4 Embryo numbers

Table 26: Embryo numbers at test end (Dienogest).

Concentrations given as geometric mean measured concentrations [ng/L].

Concentration [ng/L]	Replicate	Snail No.	Number of embryos			Length [mm]
			Shelled	Unshelled	Total	
Control	0-1	1	5	1	6	3.63
		2	1	11	12	3.53
		3	1	4	5	3.84
		4	4	6	10	3.51
		5	3	5	8	3.58
		6	5	7	12	3.52
		Mean	3.2	5.7	8.8	3.60
	0-2	1	3	2	5	3.54
		2	1	0	1	3.71
		3	4	7	11	3.51
		4	6	7	13	3.53
		5	0	5	5	3.55
		6	4	3	7	3.57
		Mean	3.0	4.0	7.0	3.57
	0-3	1	6	2	8	3.63
		2	1	1	2	3.52
		3	3	2	5	3.60
		4	2	1	3	3.50
		5	0	2	2	3.51
		6	4	6	10	3.58
		Mean	2.7	2.3	5.0	3.56
	0-4	1	2	3	5	3.54
		2	1	9	10	3.83
		3	6	2	8	3.61
		4	3	1	4	3.50
		5	5	2	7	3.53
		6	5	2	7	3.51
		Mean	3.7	3.2	6.8	3.59

Concentration [ng/L]	Replicate	Snail No.	Number of embryos			Length [mm]
	0-5	1	4	3	7	3.76
		2	9	8	17	3.90
		3	4	3	7	3.53
		4	3	4	7	3.52
		5	6	4	10	3.61
		6	2	3	5	3.64
		Mean	4.7	4.2	8.8	3.66
	0-6	1	1	2	3	3.63
		2	6	4	10	3.55
		3	2	0	2	3.73
		4	7	5	12	4.03
		5	4	3	7	3.56
		6	5	1	6	3.50
		Mean	4.2	2.5	6.7	3.67
6.97	1-1	1	1	3	4	3.76
		2	0	9	9	3.74
		3	12	7	19	3.71
		4	2	9	11	3.56
		5	5	5	10	3.50
		6	6	3	9	3.58
		Mean	4.3	6.0	10.3	3.64
	1-2	1	3	4	7	3.77
		2	7	10	17	3.81
		3	6	5	11	3.56
		4	4	5	9	3.66
		5	2	4	6	3.72
		6	0	3	3	3.50
		Mean	3.7	5.2	8.8	3.67
	1-3	1	9	7	16	3.50
		2	6	2	8	3.65
		3	1	3	4	3.51
		4	8	6	14	3.60

Concentration [ng/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		5	4	2	6	3.52
		6	0	4	4	3.64
		Mean	4.7	4.0	8.7	3.57
	1-4	1	6	3	9	3.50
		2	4	5	9	3.51
		3	3	3	6	3.50
		4	0	1	1	3.53
		5	7	6	13	3.57
		6	5	0	5	3.69
		Mean	4.2	3.0	7.2	3.55
	1-5	1	8	4	12	3.51
		2	2	3	5	3.60
		3	0	3	3	3.73
		4	1	1	2	3.50
		5	5	5	10	3.50
		6	2	5	7	3.51
		Mean	3.0	3.5	6.5	3.56
	1-6	1	7	7	14	3.88
		2	5	4	9	3.53
		3	3	7	10	3.50
		4	1	1	2	3.52
		5	5	3	8	3.51
		6	5	3	8	3.50
		Mean	4.3	4.2	8.5	3.57
11.2	2-1	1	7	3	10	3.51
		2	9	7	16	3.53
		3	6	6	12	3.86
		4	5	7	12	3.50
		5	4	0	4	3.56
		6	3	2	5	3.56
		Mean	5.7	4.2	9.8	3.59
	2-2	1	0	4	4	3.56

Concentration [ng/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		2	2	6	8	3.60
		3	8	2	10	3.60
		4	7	7	14	4.19
		5	0	2	2	3.51
		6	6	8	14	3.55
		Mean	3.8	4.8	8.7	3.67
	2-3	1	4	5	9	3.52
		2	4	4	8	3.62
		3	3	5	8	3.73
		4	1	1	2	3.63
		5	3	1	4	3.56
		6	2	4	6	3.51
		Mean	2.8	3.3	6.2	3.60
	2-4	1	7	7	14	3.60
		2	4	7	11	3.50
		3	10	8	18	3.69
		4	8	5	13	3.71
		5	2	2	4	3.71
		6	1	8	9	3.58
		Mean	5.3	6.2	11.5	3.63
	2-5	1	5	8	13	3.67
		2	6	8	14	3.56
		3	3	1	4	3.61
		4	8	10	18	3.58
		5	5	7	12	3.52
		6	4	4	8	3.52
		Mean	5.2	6.3	11.5	3.58
	2-6	1	3	4	7	3.63
		2	9	3	12	3.58
		3	1	5	6	3.50
		4	6	6	12	3.79
		5	3	3	6	3.58

Concentration [ng/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		6	1	5	6	3.50
		Mean	3.8	4.3	8.2	3.60
25.6	3-1	1	1	0	1	3.65
		2	6	7	13	3.61
		3	6	4	10	3.71
		4	10	4	14	3.58
		5	0	6	6	3.51
		6	2	5	7	3.78
		Mean	4.2	4.3	8.5	3.64
	3-2	1	6	7	13	3.54
		2	5	7	12	3.53
		3	3	5	8	3.53
		4	4	3	7	3.50
		5	0	1	1	3.59
		6	5	2	7	3.52
		Mean	3.8	4.2	8.0	3.54
	3-3	1	3	3	6	3.51
		2	7	8	15	3.68
		3	6	2	8	3.69
		4	5	6	11	3.51
		5	3	6	9	3.65
		6	7	2	9	3.67
		Mean	5.2	4.5	9.7	3.62
	3-4	1	5	4	9	3.52
		2	5	3	8	3.50
		3	3	3	6	3.59
		4	4	3	7	3.62
		5	5	3	8	3.67
		6	7	4	11	3.52
		Mean	4.8	3.3	8.2	3.57
	3-5	1	6	7	13	3.59
		2	0	1	1	3.53

Concentration [ng/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		3	6	2	8	3.56
		4	5	8	13	3.54
		5	5	5	10	3.53
		6	2	1	3	3.68
		Mean	4.0	4.0	8.0	3.57
	3-6	1	5	4	9	3.51
		2	5	5	10	3.51
		3	5	4	9	3.50
		4	5	1	6	3.52
		5	4	4	8	3.57
		6	3	3	6	3.50
		Mean	4.5	3.5	8.0	3.52
106	4-1	1	2	2	4	3.51
		2	4	4	8	3.64
		3	4	0	4	3.87
		4	5	2	7	3.50
		5	3	4	7	3.52
		6	1	7	8	3.53
		Mean	3.2	3.2	6.3	3.60
	4-2	1	6	4	10	3.50
		2	0	5	5	3.77
		3	5	5	10	3.82
		4	2	5	7	3.50
		5	3	4	7	3.64
		6	4	4	8	3.52
		Mean	3.3	4.5	7.8	3.63
	4-3	1	2	3	5	3.59
		2	4	5	9	3.50
		3	4	4	8	3.55
		4	5	5	10	3.83
		5	1	4	5	3.50
		6	3	8	11	3.61

Concentration [ng/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		Mean	3.2	4.8	8.0	3.60
	4-4	1	1	4	5	3.51
		2	2	5	7	3.68
		3	7	7	14	3.54
		4	4	4	8	3.67
		5	4	5	9	3.85
		6	0	1	1	3.62
		Mean	3.0	4.3	7.3	3.65
	4-5	1	7	4	11	3.53
		2	2	6	8	3.50
		3	3	4	7	3.52
		4	6	6	12	3.52
		5	6	2	8	3.50
		6	6	4	10	3.96
		Mean	5.0	4.3	9.3	3.59
	4-6	1	4	6	10	3.56
		2	4	3	7	3.60
		3	4	8	12	3.51
		4	10	6	16	3.85
		5	6	6	12	3.50
		6	7	7	14	3.60
		Mean	5.8	6.0	11.8	3.60
332	5-1	1	3	5	8	3.54
		2	2	3	5	3.50
		3	5	6	11	3.76
		4	7	4	11	3.63
		5	5	4	9	3.80
		6	1	0	1	3.55
		Mean	3.8	3.7	7.5	3.63
	5-2	1	8	6	14	3.51
		2	1	3	4	3.81
		3	4	3	7	3.53

Concentration [ng/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		4	6	2	8	4.03
		5	5	3	8	3.70
		6	4	3	7	3.50
		Mean	4.7	3.3	8.0	3.68
	5-3	1	4	4	8	3.50
		2	3	6	9	3.50
		3	4	4	8	3.54
		4	5	3	8	3.74
		5	4	2	6	3.55
		6	3	1	4	3.54
		Mean	3.8	3.3	7.2	3.56
	5-4	1	4	10	14	3.51
		2	4	6	10	3.52
		3	6	3	9	3.62
		4	8	3	11	3.51
		5	1	7	8	3.53
		6	5	1	6	3.74
		Mean	4.7	5.0	9.7	3.57
	5-5	1	6	5	11	3.84
		2	2	0	2	3.50
		3	4	8	12	3.59
		4	4	6	10	3.53
		5	0	1	1	3.52
		6	4	6	10	3.60
		Mean	3.3	4.3	7.7	3.60
	5-6	1	7	2	9	3.63
		2	12	9	21	3.67
		3	4	6	10	3.53
		4	1	6	7	3.51
		5	8	2	10	3.56
		6	4	5	9	3.65
		Mean	6.0	5.0	11.0	3.59

Table 27: Number of living snails per replicate at test end (Dienogest).

Concentrations given as geometric mean measured concentrations [ng/L].

Date	Replicate	Control	6.97	11.2	25.6	106	332
Day 28 Test end	1	6	6	6	6	6	6
	2	6	6	6	6	6	6
	3	6	6	6	6	6	6
	4	6	6	6	6	6	6
	5	6	6	6	6	6	6
	6	6	6	6	6	6	6

A.2 Processed raw data – Reproduction test with Dexamethason

A.2.1 Fecundity of snails before test start

Table 28: Fecundity and Length of 20 randomly selected snails before test start (Dexamethasone).

Subtitle *[if not necessary, please remove]*

	Embryo number			
Snail No.	**Shelled**	**unshelled**	**total**	**Length**
1	6	7	13	3.65
2	11	8	19	3.89
3	0	3	3	3.69
4	4	3	7	3.67
5	6	9	15	3.53
6	0	7	7	3.51
7	4	8	12	3.50
8	5	7	12	3.57
9	16	8	24	3.58
10	8	6	14	3.55
11	6	15	21	3.78
12	4	9	13	3.52
13	10	6	16	3.52
14	6	9	15	3.93
15	3	8	11	3.51
16	3	6	9	3.68
17	5	5	10	4.01
18	2	9	11	3.71
19	3	6	9	3.51
20	5	2	7	4.01
Mean	5.4	7.1	12.4	3.67

A.2.2 Water chemistry data

Table 29: Oxygen concentration of the overlying water [mg/L] during the test with Dexamethasone.

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Control		1.06		3.54		10.5		36.3		105	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	8.50	-	8.30	-	8.30	-	8.40	-	8.50	-	8.60	-
Day 2	7.80	8.80	8.00	9.10	8.50	8.90	8.50	8.90	8.50	8.90	8.80	9.00
Day 5	8.00	8.10	8.20	8.30	8.10	8.50	8.20	8.00	8.30	8.50	7.70	8.30
Day 7	7.70	7.90	8.30	8.10	8.20	8.20	8.20	8.40	8.10	8.20	8.20	8.20
Day 9	8.50	8.20	8.40	8.60	8.40	8.50	8.30	8.50	8.40	8.00	8.20	8.40
Day 12	8.00	7.90	7.90	7.70	7.50	8.20	8.10	7.90	8.20	7.80	8.30	7.70
Day 14	8.50	8.50	8.50	8.60	8.30	8.60	8.20	8.80	8.50	8.50	8.50	8.60
Day 16	8.50	8.70	7.70	8.60	8.30	8.60	8.50	8.60	8.10	8.60	8.50	8.60
Day 19	8.20	8.50	8.40	8.60	8.30	8.80	8.10	8.80	8.10	8.60	8.30	8.80
Day 21	8.60	8.80	8.50	8.40	8.60	8.80	8.60	8.80	8.50	8.60	8.60	8.80
Day 23	8.50	8.20	8.10	8.30	7.80	8.50	8.20	8.70	7.80	8.50	8.20	8.10
Day 26	8.50	8.90	8.50	8.90	8.50	9.00	8.60	9.00	8.40	8.80	8.50	9.00
Day 28	-	8.50	-	8.60	-	8.70	-	9.20	-	9.40	-	9.20

Values of the parallel test vessels throughout the test.

Table 30: Oxygen saturation of the overlying water [%] during the test with Dexamethasone.

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Control		1.06		3.54		10.5		36.3		105	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	101	-	95	-	93	-	96	-	98	-	98	-
Day 2	92	100	96	101	99	103	98	103	99	101	99	104
Day 5	91	93	94	94	92	94	94	94	94	97	88	94
Day 7	94	92	98	95	95	94	95	96	98	95	97	95
Day 9	96	92	95	98	97	96	97	96	97	92	94	97
Day 12	97	97	96	92	88	96	93	97	94	95	97	93
Day 14	97	98	97	95	97	97	97	99	97	97	97	97
Day 16	98	100	90	97	98	97	98	97	93	96	94	95
Day 19	95	97	97	98	95	100	93	100	93	100	95	100
Day 21	97	100	97	98	98	100	97	100	95	98	97	98
Day 23	95	96	92	95	90	97	94	98	89	97	93	96
Day 26	97	102	96	100	97	101	95	101	95	100	95	100
Day 28	-	98	-	97	-	99	-	103	-	105	-	104

Values of the parallel test vessels throughout the test.

Table 31: pH of the overlying water during the test with Dexamethasone.

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Control		1.06		3.54		10.5		36.3		105	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	7.71	-	7.70	-	7.74	-	7.77	-	7.78	-	7.78	-
Day 2	8.09	8.17	8.08	8.18	8.12	8.18	8.13	8.19	8.15	8.21	8.15	8.19
Day 5	8.30	8.32	8.41	8.34	8.20	8.34	8.18	8.33	8.18	8.33	8.14	8.32
Day 7	8.33	8.46	8.24	8.31	8.18	8.27	8.25	8.27	8.23	8.23	8.19	8.24
Day 9	7.91	7.91	7.97	7.93	7.84	7.98	7.99	7.98	7.98	8.03	8.02	8.03
Day 12	8.41	8.45	8.32	8.38	8.15	8.33	8.27	8.30	8.27	8.28	8.24	8.29
Day 14	8.29	8.05	8.31	8.09	8.29	8.13	8.29	8.15	8.34	8.20	8.39	8.20
Day 16	8.27	8.39	8.11	8.42	8.28	8.45	8.28	8.46	8.19	8.46	8.26	8.51
Day 19	7.98	8.12	8.00	8.14	7.99	8.15	7.96	8.14	8.01	8.18	8.02	8.19
Day 21	8.05	8.15	8.05	8.16	8.04	8.16	8.08	8.16	8.04	8.19	8.09	8.19
Day 23	8.04	8.20	8.07	8.18	7.99	8.21	8.09	8.20	7.99	8.21	8.03	8.20
Day 26	8.06	8.17	8.11	8.16	8.08	8.19	8.07	8.18	8.09	8.19	8.11	8.21
Day 28	-	8.21	-	8.22	-	8.26	-	8.24	-	8.22	-	8.22

Values of the parallel test vessels throughout the test.

Table 32: Conductivity of the overlying water [µS/cm] during the test with Dexamethasone.

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Control		1.06		3.54		10.5		36.3		105	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	735	-	736	-	735	-	736	-	735	-	736	-
Day 2	725	743	724	741	725	741	724	742	723	744	723	744
Day 5	731	735	732	733	731	734	731	734	730	736	730	736
Day 7	732	736	733	736	733	736	733	733	732	736	732	735
Day 9	764	746	757	736	757	738	758	738	758	739	758	739
Day 12	757	779	747	768	754	764	745	769	748	767	747	769
Day 14	733	747	733	745	733	744	734	744	732	745	734	744
Day 16	769	737	768	739	769	740	768	737	767	738	767	740
Day 19	756	777	753	778	755	777	756	776	753	782	753	777
Day 21	772	763	771	759	771	761	772	760	767	765	770	762
Day 23	769	776	767	773	768	776	766	776	764	775	765	775
Day 26	728	772	734	768	726	772	724	773	724	772	726	774
Day 28	-	733	-	729	-	733	-	731	-	730	-	730

Values of the parallel test vessels throughout the test.

Table 33: Weekly measurements of nitrate in the overlying water [mg/L] during the test with Dexamethasone.

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Control		1.06		3.54		10.5		36.3		105	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	2.00	-	4.00	-	3.00	-	2.00	-	2.00	-	3.00	-
Day 2	-	2.00	-	3.00	-	3.00	-	2.00	-	2.00	-	3.00
Day 7	4.00	-	5.00	-	3.00	-	5.00	-	3.00	-	3.00	-
Day 9	-	3.00	-	4.00	-	3.00	-	3.00	-	2.00	-	3.00
Day 14	5.00	-	7.00	-	6.00	-	6.00	-	6.00	-	8.00	-
Day 16	-	6.00	-	6.00	-	5.00	-	5.00	-	6.00	-	6.00
Day 21	5.00	-	5.00	-	3.00	-	5.00	-	4.00	-	5.00	-
Day 23	-	1.00	-	1.00	-	2.00	-	2.00	-	2.00	-	2.00

Values of the parallel test vessels throughout the test.

Table 34: Weekly measurements of nitrite in the overlying water [mg/L] during the test with Dexamethasone.

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Control		1.06		3.54		10.5		36.3		105	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	0.005	-	0.004	-	0.004	-	0.004	-	0.004	-	0.004	-
Day 2	-	0.018	-	0.017	-	0.019	-	0.021	-	0.020	-	0.022
Day 7	0.000	-	0.002	-	0.001	-	0.000	-	0.002	-	0.001	-
Day 9	-	0.015	-	0.016	-	0.016	-	0.013	-	0.015	-	0.017
Day 14	0.091	-	0.078	-	0.108	-	0.116	-	0.080	-	0.074	-
Day 16	-	0.022	-	0.014	-	0.019	-	0.022	-	0.013	-	0.013
Day 21	0.000	-	0.003	-	0.002	-	0.001	-	0.001	-	0.000	-
Day 23	-	0.043	-	0.036	-	0.036	-	0.040	-	0.043	-	0.043

Values of the parallel test vessels throughout the test.

Table 35: Weekly measurements of ammonia in the overlying water [mg/L] during the test with Dexamethasone.

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Control		1.06		3.54		10.5		36.3		105	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	0.02	-	0.02	-	0.01	-	0.02	-	0.01	-	0.02	-
Day 2	-	0.00	-	0.00	-	0.00	-	0.01	-	0.00	-	0.02
Day 7	0.01	-	0.00	-	0.01	-	0.01	-	0.01	-	0.01	-
Day 9	-	0.00	-	0.00	-	0.01	-	0.01	-	0.00	-	0.01
Day 14	0.00	-	0.00	-	0.01	-	0.00	-	0.00	-	0.00	-
Day 16	-	0.00	-	0.00	-	0.02	-	0.06	-	0.03	-	0.04
Day 21	0.00	-	0.02	-	0.00	-	0.00	-	0.00	-	0.00	-
Day 23	-	0.03	-	0.02	-	0.03	-	0.03	-	0.03	-	0.03

Values of the parallel test vessels throughout the test.

A.2.3 Temperature and Light

Table 36: Temperature of the overlying water [°C] during the test with Dexamethasone

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Control		1.06		3.54		10.5		36.3		105	
	new	aged	new	aged	new	aged	new	aged	new	aged	new	aged
Day 0	16.7	-	16.8	-	16.9	-	16.9	-	16.8	-	16.8	-
Day 2	16.7	16.8	16.9	16.8	17.0	16.7	17.0	16.9	17.0	16.8	16.9	16.8
Day 5	16.8	16.9	17.0	17.0	17.1	17.0	17.1	17.0	17.0	17.0	17.0	16.9
Day 7	16.9	17.2	17.0	17.5	17.1	17.4	17.2	17.3	17.2	17.5	17.1	17.5
Day 9	17.5	17.3	17.2	17.0	17.2	17.1	17.4	17.0	17.2	17.1	17.5	17.1
Day 12	17.5	17.0	17.4	17.1	17.3	17.2	17.5	17.2	17.4	17.4	17.2	17.2
Day 14	16.4	17.0	16.6	17.0	16.5	17.0	16.8	17.0	16.5	17.1	16.6	17.0
Day 16	17.3	17.0	17.4	17.0	17.4	17.0	17.4	16.9	17.4	16.9	17.3	16.9
Day 19	17.3	16.7	17.4	16.8	17.4	16.8	17.4	16.7	17.3	16.8	17.4	16.8
Day 21	17.3	16.9	17.4	16.9	17.5	16.9	17.5	17.0	17.5	16.9	17.4	17.0
Day 23	17.3	17.3	17.4	17.4	17.5	17.4	17.5	17.4	17.5	17.4	17.4	17.4
Day 26	17.3	16.8	17.5	16.8	17.5	16.9	17.5	16.8	17.4	16.9	17.3	16.9
Day 28	-	16.7	-	16.7	-	16.7	-	16.7	-	16.7	-	16.7

Table 37: Light intensity [lx] in the test chamber throughout the test during the test with Dexamethasone.

Date	Light intensity [lx
Day 0	558
Day 2	574
Day 5	524
Day 7	582
Day 9	588
Day 12	572
Day 14	569
Day 16	568
Day 19	570
Day 21	571
Day 23	544
Day 26	559
Day 28	557

A.2.4 Embryo numbers

Table 38: Embryo numbers at test end (Dexamethasone).

Concentrations given as geometric mean measured concentrations [µg/L].

Concentration [µg/L]	Replicate	Snail No.	Number of embryos			Length [mm]
			Shelled	Unshelled	Total	
Control	0-1	1	13	7	20	3.92
		2	9	9	18	3.76
		3	9	4	13	4.09
		4	4	7	11	3.70
		5	10	9	19	3.62
		6	7	9	16	3.58
		Mean	8.7	7.5	16.2	3.78
	0-2	1	11	3	14	3.71
		2	13	9	22	3.62
		3	12	7	19	3.89
		4	8	5	13	3.59
		5	9	6	15	3.76
		6	12	9	21	3.79
		Mean	10.8	6.5	17.3	3.73
	0-3	1	10	5	15	3.85
		2	13	5	18	4.28
		3	5	13	18	3.56
		4	9	7	16	3.60
		5	9	9	18	4.29
		6	10	4	14	4.27
		Mean	9.3	7.2	16.5	3.98
	0-4	1	12	2	14	3.74
		2	10	6	16	3.96
		3	10	7	17	3.53
		4	9	1	10	3.62
		5	7	7	14	3.52
		6	0	3	3	3.55
		Mean	8.0	4.3	12.3	3.65

Concentration [µg/L]	Replicate	Snail No.	Number of embryos			Length [mm]
	0-5	1	12	2	14	3.97
		2	12	9	21	4.27
		3	7	12	19	3.57
		4	7	5	12	3.66
		5	7	8	15	3.56
		6	10	1	11	3.52
		Mean	9.2	6.2	15.3	3.76
	0-6	1	7	6	13	3.54
		2	6	9	15	3.52
		3	3	8	11	3.53
		4	0	6	6	4.08
		5	8	13	21	3.71
		6	8	4	12	3.52
		Mean	5.3	7.7	13.0	3.65
1.06	1-1	1	3	11	14	3.90
		2	5	7	12	3.62
		3	5	8	13	3.50
		4	14	8	22	3.82
		5	0	5	5	3.54
		6	6	9	15	3.52
		Mean	5.5	8.0	13.5	3.65
	1-2	1	6	8	14	3.70
		2	7	10	17	3.98
		3	11	8	19	3.66
		4	5	4	9	3.55
		5	5	11	16	3.57
		6	9	9	18	3.94
		Mean	7.2	8.3	15.5	3.73
	1-3	1	5	8	13	3.51
		2	6	7	13	3.61
		3	11	4	15	3.68
		4	6	6	12	3.77

Concentration [µg/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		5	7	5	12	3.64
		6	5	7	12	3.77
		Mean	6.7	6.2	12.8	3.66
	1-4	1	11	7	18	3.50
		2	7	5	12	3.74
		3	8	11	19	3.53
		4	9	9	18	3.95
		5	5	6	11	3.90
		6	8	10	18	3.68
		Mean	8.0	8.0	16.0	3.72
	1-5	1	12	9	21	4.38
		2	11	7	18	3.61
		3	2	6	8	3.63
		4	7	8	15	3.57
		5	8	8	16	3.56
		6	0	3	3	3.52
		Mean	6.7	6.8	13.5	3.71
	1-6	1	2	26	28	3.54
		2	8	10	18	3.57
		3	5	0	5	3.71
		4	7	11	18	4.04
		5	3	4	7	3.58
		6	3	6	9	3.61
		Mean	4.7	9.5	14.2	3.68
3.54	2-1	1	4	8	12	3.86
		2	0	7	7	3.75
		3	5	7	12	3.67
		4	10	10	20	3.57
		5	8	7	15	3.58
		6	9	8	17	4.24
		Mean	6.0	7.8	13.8	3.78
	2-2	1	1	3	4	3.93

Concentration [µg/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		2	6	7	13	3.58
		3	9	6	15	3.73
		4	8	3	11	3.69
		5	7	7	14	3.51
		6	7	6	13	3.53
		Mean	6.3	5.3	11.7	3.66
	2-3	1	10	2	12	3.51
		2	5	5	10	3.50
		3	0	4	4	3.99
		4	6	9	15	3.53
		5	9	10	19	3.68
		6	0	0	0	3.61
		Mean	5.0	5.0	10.0	3.64
	2-4	1	6	10	16	3.52
		2	6	7	13	3.51
		3	4	8	12	3.53
		4	5	9	14	3.60
		5	9	8	17	3.74
		6	8	4	12	3.81
		Mean	6.3	7.7	14.0	3.62
	2-5	1	6	6	12	3.63
		2	5	13	18	3.55
		3	8	8	16	3.69
		4	7	5	12	3.52
		5	6	7	13	3.60
		6	5	9	14	3.58
		Mean	6.2	8.0	14.2	3.60
	2-6	1	7	4	11	3.65
		2	4	9	13	3.65
		3	8	2	10	3.52
		4	9	2	11	3.61
		5	7	6	13	3.71

Concentration [µg/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		6	2	4	6	4.16
		Mean	6.2	4.5	10.7	3.72
10.5	3-1	1	4	10	14	3.94
		2	0	3	3	3.56
		3	8	9	17	3.80
		4	6	5	11	3.71
		5	11	11	22	3.63
		6	6	14	20	4.20
		Mean	5.8	8.7	14.5	3.81
	3-2	1	12	8	20	3.75
		2	10	12	22	4.24
		3	7	6	13	3.68
		4	8	7	15	3.83
		5	9	8	17	3.74
		6	5	7	12	3.53
		Mean	8.5	8.0	16.5	3.80
	3-3	1	12	6	18	4.17
		2	6	8	14	3.50
		3	12	8	20	3.61
		4	8	4	12	3.56
		5	5	9	14	3.61
		6	6	9	15	3.56
		Mean	8.2	7.3	15.5	3.67
	3-4	1	9	8	17	3.55
		2	8	9	17	3.50
		3	9	6	15	3.57
		4	8	6	14	3.79
		5	5	10	15	3.71
		6	4	6	10	3.64
		Mean	7.2	7.5	14.7	3.63
	3-5	1	12	5	17	4.25
		2	13	9	22	3.64

Concentration [µg/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		3	10	7	17	3.52
		4	10	9	19	3.50
		5	5	13	18	3.60
		6	8	7	15	3.70
		Mean	9.7	8.3	18.0	3.70
	3-6	1	9	6	15	3.85
		2	7	5	12	3.59
		3	13	8	21	3.52
		4	9	9	18	3.98
		5	5	14	19	3.57
		6	3	6	9	3.96
		Mean	7.7	8.0	15.7	3.75
36.3	4-1	1	4	10	14	3.90
		2	6	11	17	3.67
		3	8	6	14	3.54
		4	5	10	15	3.54
		5	4	5	9	3.96
		6	11	10	21	4.14
		Mean	6.3	8.7	15.0	3.79
	4-2	1	6	10	16	3.58
		2	7	2	9	3.59
		3	8	6	14	3.82
		4	9	5	14	3.54
		5	5	14	19	3.70
		6	12	5	17	3.59
		Mean	7.8	7.0	14.8	3.64
	4-3	1	0	8	8	4.12
		2	1	4	5	3.52
		3	8	7	15	3.50
		4	2	10	12	3.89
		5	7	12	19	3.52
		6	9	9	18	4.05

Concentration [µg/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		Mean	4.5	8.3	12.8	3.77
	4-4	1	6	10	16	4.07
		2	5	6	11	3.62
		3	7	9	16	3.64
		4	12	11	23	3.89
		5	12	8	20	4.26
		6	5	7	12	3.51
		Mean	7.8	8.5	16.3	3.83
	4-5	1	5	9	14	3.92
		2	2	9	11	4.14
		3	9	7	16	3.56
		4	7	8	15	3.64
		5	11	12	23	4.43
		6	14	7	21	3.78
		Mean	8.0	8.7	16.7	3.91
	4-6	1	8	9	17	3.95
		2	8	9	17	4.05
		3	8	5	13	3.52
		4	8	6	14	3.78
		5	8	11	19	3.53
		6	8	6	14	3.50
		Mean	8.0	7.7	15.7	3.72
105	5-1	1	2	4	6	3.94
		2	5	3	8	3.63
		3	4	7	11	3.56
		4	9	7	16	3.54
		5	7	8	15	3.56
		6	10	5	15	3.58
		Mean	6.2	5.7	11.8	3.64
	5-2	1	5	8	13	3.52
		2	4	2	6	3.59
		3	0	6	6	3.83

Concentration [µg/L]	Replicate	Snail No.	Number of embryos			Length [mm]
		4	2	8	10	3.72
		5	13	5	18	4.07
		6	9	12	21	3.76
		Mean	5.5	6.8	12.3	3.75
	5-3	1	9	8	17	3.56
		2	9	8	17	3.56
		3	4	11	15	3.55
		4	6	4	10	3.57
		5	4	4	8	3.87
		6	11	10	21	3.55
		Mean	7.2	7.5	14.7	3.61
	5-4	1	3	12	15	3.86
		2	11	11	22	3.52
		3	0	4	4	3.54
		4	1	9	10	3.56
		5	0	0	0	3.59
		6	10	9	19	3.57
		Mean	4.2	7.5	11.7	3.61
	5-5	1	4	13	17	3.97
		2	0	3	3	3.52
		3	0	0	0	3.92
		4	7	9	16	3.53
		5	6	8	14	3.87
		6	7	9	16	3.81
		Mean	4.0	7.0	11.0	3.77
	5-6	1	10	10	20	3.53
		2	8	6	14	3.63
		3	8	12	20	3.78
		4	6	6	12	4.39
		5	7	9	16	3.66
		6	7	6	13	3.73
		Mean	7.7	8.2	15.8	3.79

Table 39: Number of living snails per replicate at test end (Dexamethasone).

Concentrations given as geometric mean measured concentrations [µg/L].

Date	Replicate	Control	1.06	3.54	10.5	36.3	105
Day 28 Test end	1	6	6	6	6	6	6
	2	6	6	6	6	6	6
	3	6	6	6	6	6	6
	4	6	6	6	6	6	6
	5	6	6	6	6	6	6
	6	6	6	6	6	6	6

B Appendix

B.1 Statistical evaluations – Reproduction test with Dienogest

All statistical calculations were based on measured geometric mean measured concentrations of Dienogest. All results and explanations are directly imported from ToxRat (6).

B.1.1 Survival

Statistical Evaluation of a Quantal Response: Snail Reproduction test with Dienogest

General:	
Test identification/project no.	Snail Reproduction test with Dienogest
Test item	Dienogest
Unit of test item concentration	ng/L
Start of experiment on day	10/7/2019
Date and time of the evaluation	21/11/2019; 08:28:29
Raw data filename:	ToxRat Auswertung Mortality_raw data.xls
Test design	
Number of treatments (incl. control(s))	6
Duration of the test	28 d
Measurement variable	Mortality
Test system	Potamopyrgus antipodarum

Statistical Results on the Mortality of Potamopyrgus antipodarum

Mortality of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Mortality of Potamopyrgus antipodarum as dependent on concentration of the test item and time (from InputRawData)

Treatm. [ng/L]	Control	6.970	11.170	25.600	106.000	332.000
0 d	6	6	6	6	6	6
	6	6	6	6	6	6
	6	6	6	6	6	6
	6	6	6	6	6	6
	6	6	6	6	6	6
	6	6	6	6	6	6
Total Introduced	36	36	36	36	36	36
n:	6	6	6	6	6	6
28 d	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
Total Dead:	0	0	0	0	0	0
n:	6	6	6	6	6	6

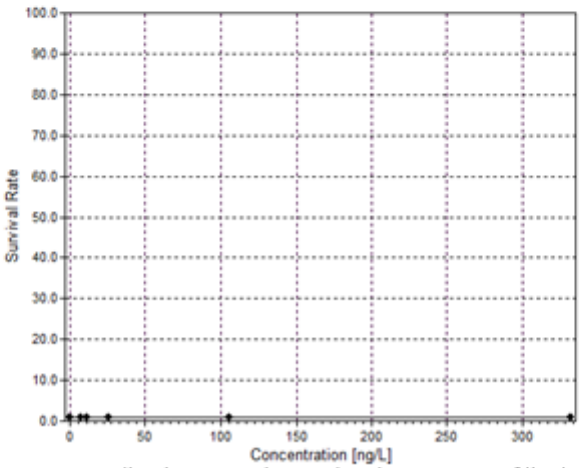


Fig. 1: Mortality of Potamopyrgus antipodarum as observed under presence of the test item after 28 d.

Lethal Concentrations (LCx) for Mortality at 28 d

Overview Mortality

Tab. 2: Overview Mortality: Overview over the effects on mortality in Potamopyrgus antipodarum at 28 d

Treatm.[ng/L]	Total Introduced	Survived	Dead	% Mortality
Control	36	36	0	0.000
6.970	36	36	0	0.000
11.170	36	36	0	0.000
25.600	36	36	0	0.000
106.000	36	36	0	0.000
332.000	36	36	0	0.000

Because no change in mortality was to be observed, no further computations have been performed for 28 d.

Because no change in mortality was to be observed, no further computations have been performed for 28 d.

Threshold concentrations (NOEC) for Mortality at 28 d

To justify the use of the Step-down Cochran-Armitage test at first a trend analysis by contrasts using proportions was performed.

Qualitative Trend Analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 2: Qualitative trend analysis by contrasts (monotonicity of concentration/response) with mortality at 28 d: Psi: total of proportions weighted by contrasts; Var(psi): variance of psi; df: degrees of freedom; Chi²: Chi²-statistic; p(Chi²): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	Var(psi)	df	Chi²	p(Chi²)
Linear	0.0000	0.0000	5	NAN	1.000
Quadratic	0.0000	0.0000	5	NAN	1.000

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, nonetheless the user-selected Step-down Cochran-Armitage test was performed.

Ahead of the Cochran-Armitage test Tarone´s test had to be performed to test for extra-binomial variance.

Tarone´s Test Procedure

Tab. 3: Tarone Test with mortality at 28 d: Treatment-wise testing the homgeneity of proportions (Alpha = 0.010). The statistic TZ has an asymptotic chi² distribution with one degree of freedom and measures the deviation from homogeneity. Ho (Phi = 0; i.e. homogeneity) is accepted, if the probability p(TZ) > Alpha; p(TZ) is the probability that the deviation from homogeneity observed in the treatment(s) is due to chance.

Treatm.[ng/L]	Introduced	Survived	Dead	TZp(TZ)	sign.
Control	36	36	0	3.6000.058	-
6.970	36	36	0	3.6000.058	-
11.170	36	36	0	3.6000.058	-
25.600	36	36	0	3.6000.058	-
106.000	36	36	0	3.6000.058	-
332.000	36	36	0	3.6000.058	-

+: significant; -: non-significant

In treatments no signs of extra-bionmial variance were found.

Step-down Cochran-Armitage Test Procedure

Tab. 4: Step-down Cochran-Armitage Test Procedure with mortality at 28 d: Step-down test to detect an increasing trend in responses (Alpha is 0.050; one-sided greater); Chi^2(tot): total (Pearson) Chi^2; z(trend): standardized one-sided deviation due to the linear upward trend; Chi^2(err): unexplained component of Chi^2(tot); p(tot|trend|err): probabilities that the observed results could be due to chance; Ho (no trend) is accepted, if p(trend) > Alpha. Note that the step-down test terminates after the first non-significant treatment is encountered

Treatm. [ng/L]	Total	Introduced	Dead%	Mortality	Chi^2(tot)	p(tot)	Chi^2(err)	p(err)	|z|(trend)	p(trend) Sign.
Control	36	0	0.000							
6.970	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000	-
11.170	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000	-
25.600	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000	-
106.000	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000	-
332.000	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 332.000 ng/L.

B.1.2 Length

Evaluation of a Metric Response: Snail Reproduction test with Dienogest

General:

Test identification/project no.	Snail Reproduction test with Dienogest
Test item	Dienogest
Unit of test item concentration	ng/L
Start of experiment on day	10/7/2019
Date and time of the evaluation	21/11/2019; 08:24:29
Raw data filename:	ToxRat Auswertung Length_raw data.xls

Test design

Number of treatments (incl. control(s))	6
Duration of the test	28.0 d
Measurement variable	Length at test end
Test system	Potamopyrgus antipodarum

Length at test end of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Length at test end [mm] of Potamopyrgus antipodarum as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (from Tabs Length at test end)

Treatm. [ng/L]	Control	6.970	11.170	25.600	106.000	332.000
28.0 d	3.60	3.64	3.59	3.64	3.60	3.63
	3.57	3.67	3.67	3.54	3.63	3.68
	3.56	3.57	3.60	3.62	3.60	3.56
	3.59	3.55	3.63	3.57	3.65	3.57
	3.66	3.56	3.58	3.57	3.59	3.60
	3.67	3.57	3.60	3.52	3.60	3.59
Mean:	3.61	3.59	3.61	3.58	3.61	3.60
Std.Dev.:	0.046	0.049	0.033	0.046	0.023	0.044
n:	6	6	6	6	6	6
CV:	1.281	1.371	0.917	1.283	0.641	1.225

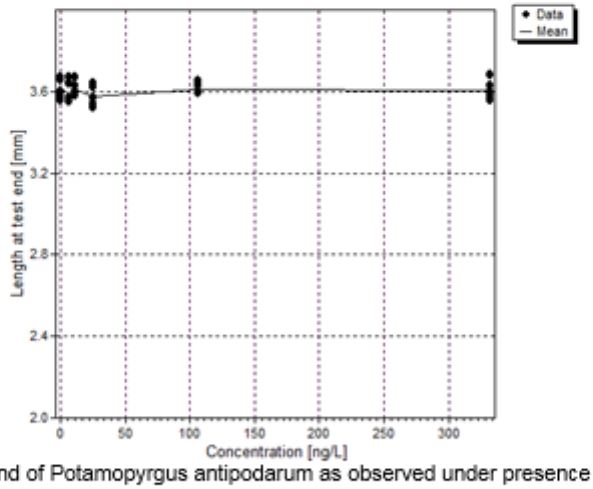


Fig. 1: Length at test end of Potamopyrgus antipodarum as observed under presence of the test item after 28.0 d.

Effective Concentrations (ECx) for Length at test end at 28.0 d

Length at test end [mm] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Increase of length at test end caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Increase
Control	3.61	0.046	6	
6.970	3.59	0.049	6	-0.416
11.170	3.61	0.033	6	0.092
25.600	3.58	0.046	6	-0.878
106.000	3.61	0.023	6	0.092
332.000	3.60	0.044	6	-0.092

Probit analysis using linear max. likelihood regression

Tab. 3: Probit analysis using linear max. likelihood regression with length at test end at 28.0 d: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [ng/L]	Log(x)	%Decrease	n	Emp. Probit	Weight	Reg. Probit
6.970	0.843	0.00	1	-1.275	0.217	-1.235
6.970	0.843	0.00	1	-1.296	0.217	-1.235
6.970	0.843	1.06	1	-1.227	0.217	-1.235
6.970	0.843	1.62	1	-1.213	0.217	-1.235
6.970	0.843	1.34	1	-1.220	0.217	-1.235
6.970	0.843	1.06	1	-1.227	0.217	-1.235
11.170	1.048	0.51	1	-1.241	0.217	-1.236
11.170	1.048	0.00	1	-1.296	0.217	-1.236
11.170	1.048	0.23	1	-1.248	0.217	-1.236
11.170	1.048	0.00	1	-1.268	0.217	-1.236
11.170	1.048	0.79	1	-1.234	0.217	-1.236
11.170	1.048	0.23	1	-1.248	0.217	-1.236
25.600	1.408	0.00	1	-1.275	0.216	-1.238
25.600	1.408	1.89	1	-1.206	0.216	-1.238
25.600	1.408	0.00	1	-1.261	0.216	-1.238
25.600	1.408	1.06	1	-1.227	0.216	-1.238
25.600	1.408	1.06	1	-1.227	0.216	-1.238
25.600	1.408	2.45	1	-1.192	0.216	-1.238
106.000	2.025	0.23	1	-1.248	0.215	-1.241
106.000	2.025	0.00	1	-1.268	0.215	-1.241
106.000	2.025	0.23	1	-1.248	0.215	-1.241
106.000	2.025	0.00	1	-1.282	0.215	-1.241
106.000	2.025	0.51	1	-1.241	0.215	-1.241
106.000	2.025	0.23	1	-1.248	0.215	-1.241

332.000	2.521	0.00	1	-1.268	0.213	-1.243
332.000	2.521	0.00	1	-1.303	0.213	-1.243
332.000	2.521	1.34	1	-1.220	0.213	-1.243
332.000	2.521	1.06	1	-1.227	0.213	-1.243
332.000	2.521	0.23	1	-1.248	0.213	-1.243
332.000	2.521	0.51	1	-1.241	0.213	-1.243

excluded: value not in line with the chosen function

Inhibition greater than 100 or lower than 0% were replaced by 100.0% and 0%, respectively.

Parameters of the probit analysis

Tab. 4: Parameters of the probit analysis with length at test end at 28.0 d: Results of the regression analysis

Parameter	Value
Computation runs:	2
Slope b:	-0.01287
Intercept a:	-1.76828
Variance of b:	0.39965
Goodness of Fit	
Chi²:	0.00777
Degrees of freedom:	28
p(Chi²):	1.000
Log EC50:	-137.42595
SE Log EC50:	6,828.81776
g-Criterion:	2.80999
Residual Variance (Chi²/df):	0.00028
r²:	0.051
F:	1.493
p(F) (df: 1;28):	0.232

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0.100 data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance). The coefficient of determination, r² (0 <= r² <= 1), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No statistically significant concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significantly different from zero).
Due to the lacking concentration/response the shown ECx could not be valid.
Nonetheless, the user decided to show the ECx, confidence limits and graphs if possible.

Results of the probit analysis

Tab. 5: Results of the probit analysis with length at test end at 28.0 d: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller's theorem).

Toxicity Metric	EC10	EC20	EC50
Value [ng/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 0.000 Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

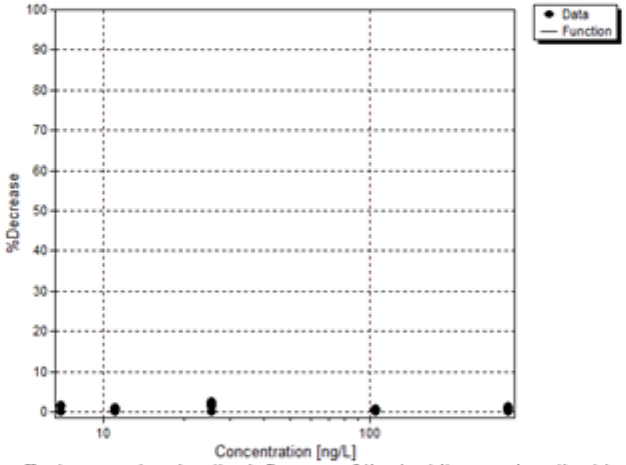


Fig. 2: Concentration-effect curve showing the influence of the test item on length at test end of the introduced Potamopyrgus antipodarum as observed after 28.0 d

Threshold concentrations (NOEC) for Length at test end at 28.0 d

Length at test end [mm] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Increase of length at test end caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Increase
Control	3.61	0.046	6	
6.970	3.59	0.049	6	-0.416
11.170	3.61	0.033	6	0.092
25.600	3.58	0.046	6	-0.878
106.000	3.61	0.023	6	0.092
332.000	3.60	0.044	6	-0.092

Statistical Characteristics of the Samples

Tab. 3: Statistical characteristics with length at test end at 28.0 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [ng/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	3.61	3.59	3.56	3.67	6	0.046	1.3	0.019	0.5	3.56	3.66
6.970	3.59	3.57	3.55	3.67	6	0.049	1.4	0.020	0.6	3.54	3.65
11.170	3.61	3.60	3.58	3.67	6	0.033	0.9	0.014	0.4	3.58	3.65
25.600	3.58	3.57	3.52	3.64	6	0.046	1.3	0.019	0.5	3.53	3.62
106.000	3.61	3.60	3.59	3.65	6	0.023	0.6	0.009	0.3	3.59	3.64
332.000	3.60	3.59	3.56	3.68	6	0.044	1.2	0.018	0.5	3.56	3.65

Shapiro-Wilk´s Test on Normal Distribution

Tab. 4: Shapiro-Wilk´s Test on Normal Distribution with length at test end at 28.0 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [ng/L]	Mean	s	n
Control	3.61	0.046	6
6.970	3.59	0.049	6
11.170	3.61	0.033	6
25.600	3.58	0.046	6
106.000	3.61	0.023	6
332.000	3.60	0.044	6

Results:

Number of residuals = 28; Shapiro-Wilk´s W = 0.904; p(W) = 0.015; p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 5: Levene´s Test on Variance Homogeneity (with Residuals) with length at test end at 28.0 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	0.0021	5	0.0004	1.011	0.428
Residuals	0.0123	30	0.0004		
Total	0.014	35			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 6: Trend analysis by contrasts (monotonicity of concentration/response) with length at test end at 28.0 d: Psi: sum of means weighted by contrasts; s(psi): standard error of psi; df: degrees of freedom; t: t-statistic; p(t): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	s(psi)	df	t	p(t)
Linear	0.0033	0.1412	30	0.024	0.491
Quadratic	0.1083	0.1547	30	0.700	0.245

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, thus the selected Williams test was replaced by Dunnett test.

Dunnett`s Multiple t-test Procedure

Tab. 7: Dunnett´s multiple t-test procedure with length at test end at 28.0 d: Comparison of treatments with "Control". Significance was Alpha = 0.050, one-sided smaller (multiple level); Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [ng/L]	Mean	s	df	%MDD	t	t*	Sign.
Control	3.61	0.0413					
6.970	3.59	0.0413	30	-1.548	-0.63	-2.34	-
11.170	3.61	0.0413	30	-1.548	0.14	-2.34	-
25.600	3.58	0.0413	30	-1.548	-1.33	-2.34	-
106.000	3.61	0.0413	30	-1.548	0.14	-2.34	-
332.000	3.60	0.0413	30	-1.548	-0.14	-2.34	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 332.000 ng/L.

B.1.3 Offspring number – Total embryos

Evaluation of a Metric Response: Snail Reproduction test with Dienogest

General:	
Test identification/project no.	Snail Reproduction test with Dienogest
Test item	Dienogest
Unit of test item concentration	ng/L
Start of experiment on day	10/7/2019
Date and time of the evaluation	21/11/2019; 08:37:36
Raw data filename:	ToxRat Auswertung Reproduction_total embryos_raw data.xls
Test design	
Number of treatments (incl. control(s))	6
Duration of the test	28.0 d
Measurement variable	Reproduction
Test system	Potamopyrgus antipodarum

Reproduction of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Reproduction [embryo number] of Potamopyrgus antipodarum as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (from Tabs Reproduction)

Treatm. [ng/L]	Control	6.970	11.170	25.600	106.000	332.000
28.0 d	8.80	10.30	9.80	8.50	6.30	7.50
	7.00	8.80	8.70	8.00	7.80	8.00
	5.00	8.70	6.20	9.70	8.00	7.20
	6.80	7.20	11.50	8.20	7.30	9.70
	8.80	6.50	11.50	8.00	9.30	7.70
	6.70	8.50	8.20	8.00	11.80	11.00
Mean:	7.18	8.33	9.32	8.40	8.42	8.52
Std.Dev.:	1.443	1.334	2.055	0.666	1.924	1.501
n:	6	6	6	6	6	6
CV:	20.085	16.004	22.054	7.933	22.859	17.627

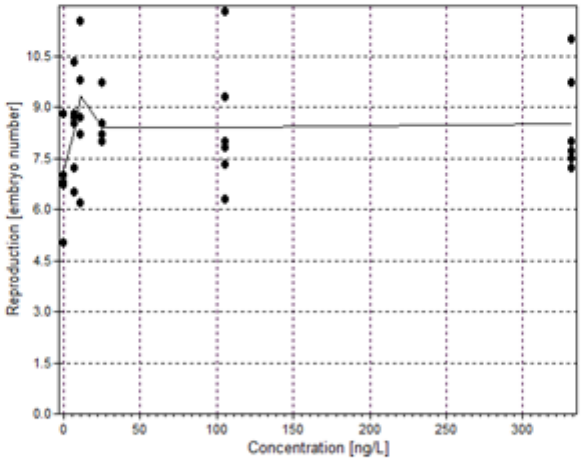


Fig. 1: Reproduction of Potamopyrgus antipodarum as observed under presence of the test item after 28.0 d.

Effective Concentrations (ECx) for Reproduction at 28.0 d

Reproduction [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Increase of reproduction caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Increase
Control	7.18	1.443	6	
6.970	8.33	1.334	6	16.009
11.170	9.32	2.055	6	29.698
25.600	8.40	0.666	6	16.937
106.000	8.42	1.924	6	17.169
332.000	8.52	1.501	6	18.561

Probit analysis using linear max. likelihood regression

Tab. 3: Probit analysis using linear max. likelihood regression with reproduction at 28.0 d: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [ng/L]	Log(x)	%Decrease	n	Emp. Probit	Weight	Reg. Probit
6.970	0.843	0.00	1	-2.341	0.231	-1.211
6.970	0.843	0.00	1	-1.817	0.231	-1.211
6.970	0.843	0.00	1	-1.783	0.231	-1.211
6.970	0.843	0.00	1	-1.259	0.231	-1.211
6.970	0.843	9.51	1	-1.015	0.231	-1.211
6.970	0.843	0.00	1	-1.713	0.231	-1.211
11.170	1.048	0.00	1	-2.166	0.229	-1.214
11.170	1.048	0.00	1	-1.783	0.229	-1.214
11.170	1.048	13.69	1	-0.910	0.229	-1.214
11.170	1.048	0.00	1	-2.760	0.229	-1.214
11.170	1.048	0.00	1	-2.760	0.229	-1.214
11.170	1.048	0.00	1	-1.608	0.229	-1.214
25.600	1.408	0.00	1	-1.713	0.225	-1.221
25.600	1.408	0.00	1	-1.538	0.225	-1.221
25.600	1.408	0.00	1	-2.132	0.225	-1.221
25.600	1.408	0.00	1	-1.608	0.225	-1.221
25.600	1.408	0.00	1	-1.538	0.225	-1.221
25.600	1.408	0.00	1	-1.538	0.225	-1.221
106.000	2.025	12.30	1	-0.945	0.219	-1.232
106.000	2.025	0.00	1	-1.469	0.219	-1.232
106.000	2.025	0.00	1	-1.538	0.219	-1.232
106.000	2.025	0.00	1	-1.294	0.219	-1.232
106.000	2.025	0.00	1	-1.992	0.219	-1.232
106.000	2.025	0.00	1	-2.864	0.219	-1.232

332.000	2.521	0.00	1	-1.364	0.214	-1.241
332.000	2.521	0.00	1	-1.538	0.214	-1.241
332.000	2.521	0.00	1	-1.259	0.214	-1.241
332.000	2.521	0.00	1	-2.132	0.214	-1.241
332.000	2.521	0.00	1	-1.434	0.214	-1.241
332.000	2.521	0.00	1	-2.585	0.214	-1.241

excluded: value not in line with the chosen function

Inhibition greater than 100 or lower than 0% were replaced by 100.0% and 0%, respectively.

Parameters of the probit analysis

Tab. 4: Parameters of the probit analysis with reproduction at 28.0 d: Results of the regression analysis

Parameter	Value
Computation runs:	2
Slope b:	-0.05011
Intercept a:	-1.66852
Variance of b:	0.38827
Goodness of Fit	
Chi²:	0.23938
Degrees of freedom:	28
p(Chi²):	1.000
Log EC50:	-33.29644
SE Log EC50:	433.40036
g-Criterion:	5.54676
Residual Variance (Chi²/df):	0.00855
r²:	0.026
F:	0.756
p(F) (df: 1;28):	0.392

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0.100 data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No statistically significant concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significantly different from zero).
Due to the lacking concentration/response the shown ECx could not be valid.
Nonetheless, the user decided to show the ECx, confidence limits and graphs if possible.

Results of the probit analysis

Tab. 5: Results of the probit analysis with reproduction at 28.0 d: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	EC10	EC20	EC50
Value [ng/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 0.000 Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

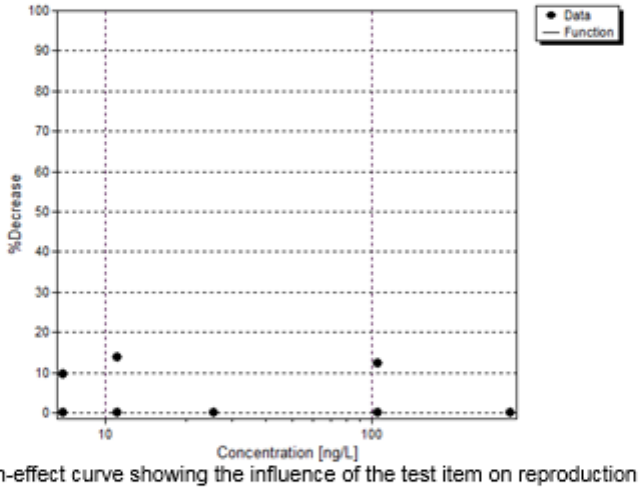


Fig. 2: Concentration-effect curve showing the influence of the test item on reproduction of the introduced Potamopyrgus antipodarum as observed after 28.0 d

Threshold concentrations (NOEC) for Reproduction at 28.0 d

Reproduction [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Increase of reproduction caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Increase
Control	7.18	1.443	6	
6.970	8.33	1.334	6	16.009
11.170	9.32	2.055	6	29.698
25.600	8.40	0.666	6	16.937
106.000	8.42	1.924	6	17.169
332.000	8.52	1.501	6	18.561

Statistical Characteristics of the Samples

Tab. 3: Statistical characteristics with reproduction at 28.0 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [ng/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	7.18	6.90	5.00	8.80	6	1.443	20.1	0.589	8.2	5.67	8.70
6.970	8.33	8.60	6.50	10.30	6	1.334	16.0	0.544	6.5	6.93	9.73
11.170	9.32	9.25	6.20	11.50	6	2.055	22.1	0.839	9.0	7.16	11.47
25.600	8.40	8.10	8.00	9.70	6	0.666	7.9	0.272	3.2	7.70	9.10
106.000	8.42	7.90	6.30	11.80	6	1.924	22.9	0.785	9.3	6.40	10.44
332.000	8.52	7.85	7.20	11.00	6	1.501	17.6	0.613	7.2	6.94	10.09

Shapiro-Wilk´s Test on Normal Distribution

Tab. 4: Shapiro-Wilk´s Test on Normal Distribution with reproduction at 28.0 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [ng/L]	Mean	s	n
Control	7.18	1.443	6
6.970	8.33	1.334	6
11.170	9.32	2.055	6
25.600	8.40	0.666	6
106.000	8.42	1.924	6
332.000	8.52	1.501	6

Results:

Number of residuals = 30; Shapiro-Wilk´s W = 0.985; p(W) = 0.937; p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 5: Levene´s Test on Variance Homogeneity (with Residuals) with reproduction at 28.0 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	4.7602	5	0.9520	1.330	0.279
Residuals	21.4820	30	0.7161		
Total	26.242	35			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 6: Trend analysis by contrasts (monotonicity of concentration/response) with reproduction at 28.0 d: Psi: sum of means weighted by contrasts; s(psi): standard error of psi; df: degrees of freedom; t: t-statistic; p(t): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	s(psi)	df	t	p(t)
Linear	6.0000	5.3064	30	1.131	0.134
Quadratic	-9.1167	5.8129	30	-1.568	0.064

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, thus the selected Williams test was replaced by Dunnett test.

Dunnett`s Multiple t-test Procedure

Tab. 7: Dunnett´s multiple t-test procedure with reproduction at 28.0 d: Comparison of treatments with "Control". Significance was Alpha = 0.050, one-sided smaller (multiple level); Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [ng/L]	Mean	s	df	%MDD	t	t*	Sign.
Control	7.18	1.5536					
6.970	8.33	1.5536	30	-29.218	1.28	-2.34	-
11.170	9.32	1.5536	30	-29.218	2.38	-2.34	-
25.600	8.40	1.5536	30	-29.218	1.36	-2.34	-
106.000	8.42	1.5536	30	-29.218	1.38	-2.34	-
332.000	8.52	1.5536	30	-29.218	1.49	-2.34	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 332.000 ng/L.

B.1.4 Offspring number – shelled embryos

Evaluation of a Metric Response: Snail Reproduction test with Dienogest

General:

Test identification/project no.	Snail Reproduction test with Dienogest
Test item	Dienogest
Unit of test item concentration	ng/L
Start of experiment on day	10/7/2019
Date and time of the evaluation	21/11/2019; 08:33:20
Raw data filename: data.xls	ToxRat Auswertung Reproduction_shelled embryos_raw

Test design

Number of treatments (incl. control(s))	6
Duration of the test	28.0 d
Measurement variable	Reproduction - shelled embryos
Test system	Potamopyrgus antipodarum

Reproduction - shelled embryos of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Reproduction - shelled embryos [embryo number] of Potamopyrgus antipodarum as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (from Tabs Reproduction - shelled embryos)

Treatm. [ng/L]	Control	6.970	11.170	25.600	106.000	332.000
28.0 d	3.20	4.30	5.70	4.20	3.20	3.80
	3.00	3.70	3.80	3.80	3.30	4.70
	2.70	4.70	2.80	5.20	3.20	3.80
	3.70	4.20	5.30	4.80	3.00	4.70
	4.70	3.00	5.20	4.00	5.00	3.30
	4.20	4.30	3.80	4.50	5.80	6.00
Mean:	3.58	4.03	4.43	4.42	3.92	4.38
Std.Dev.:	0.763	0.599	1.133	0.523	1.181	0.966
n:	6	6	6	6	6	6
CV:	21.284	14.848	25.546	11.844	30.141	22.044

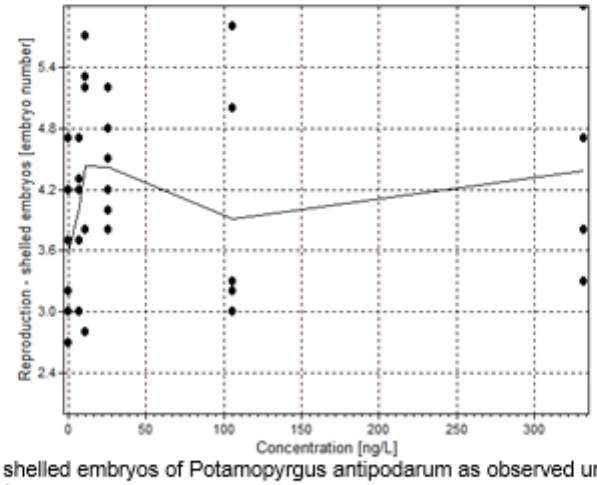


Fig. 1: Reproduction - shelled embryos of Potamopyrgus antipodarum as observed under presence of the test item after 28.0 d.

Effective Concentrations (ECx) for Reproduction - shelled embryos at 28.0 d

Reproduction - shelled embryos [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Increase of reproduction - shelled embryos caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Increase
Control	3.58	0.763	6	
6.970	4.03	0.599	6	12.558
11.170	4.43	1.133	6	23.721
25.600	4.42	0.523	6	23.256
106.000	3.92	1.181	6	9.302
332.000	4.38	0.966	6	22.326

Probit analysis using linear max. likelihood regression

Tab. 3: Probit analysis using linear max. likelihood regression with reproduction - shelled embryos at 28.0 d: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [ng/L]	Log(x)	%Decrease	n	Emp. Probit	Weight	Reg. Probit
6.970	0.843	0.00	1	-1.755	0.245	-1.185
6.970	0.843	0.00	1	-1.335	0.245	-1.185
6.970	0.843	0.00	1	-2.034	0.245	-1.185
6.970	0.843	0.00	1	-1.685	0.245	-1.185
6.970	0.843	16.28	1	-0.845	0.245	-1.185
6.970	0.843	0.00	1	-1.755	0.245	-1.185
11.170	1.048	0.00	1	-2.734	0.247	-1.183
11.170	1.048	0.00	1	-1.405	0.247	-1.183
11.170	1.048	21.86	1	-0.705	0.247	-1.183
11.170	1.048	0.00	1	-2.454	0.247	-1.183
11.170	1.048	0.00	1	-2.384	0.247	-1.183
11.170	1.048	0.00	1	-1.405	0.247	-1.183
25.600	1.408	0.00	1	-1.685	0.249	-1.179
25.600	1.408	0.00	1	-1.405	0.249	-1.179
25.600	1.408	0.00	1	-2.384	0.249	-1.179
25.600	1.408	0.00	1	-2.104	0.249	-1.179
25.600	1.408	0.00	1	-1.545	0.249	-1.179
25.600	1.408	0.00	1	-1.895	0.249	-1.179
106.000	2.025	10.70	1	-0.985	0.253	-1.172
106.000	2.025	7.91	1	-1.055	0.253	-1.172
106.000	2.025	10.70	1	-0.985	0.253	-1.172
106.000	2.025	16.28	1	-0.845	0.253	-1.172
106.000	2.025	0.00	1	-2.244	0.253	-1.172
106.000	2.025	0.00	1	-2.804	0.253	-1.172

332.000	2.521	0.00	1	-1.405	0.256	-1.167
332.000	2.521	0.00	1	-2.034	0.256	-1.167
332.000	2.521	0.00	1	-1.405	0.256	-1.167
332.000	2.521	0.00	1	-2.034	0.256	-1.167
332.000	2.521	7.91	1	-1.055	0.256	-1.167
332.000	2.521	0.00	1	-2.944	0.256	-1.167

excluded: value not in line with the chosen function

Inhibition greater than 100 or lower than 0% were replaced by 100.0% and 0%, respectively.

Parameters of the probit analysis

Tab. 4: Parameters of the probit analysis with reproduction - shelled embryos at 28.0 d: Results of the regression analysis

Parameter	Value
Computation runs:	2
Slope b:	0.02734
Intercept a:	-1.66642
Variance of b:	0.34190
Goodness of Fit	
Chi²:	0.67843
Degrees of freedom:	28
p(Chi²):	1.000
Log EC50:	60.94160
SE Log EC50:	1,269.44594
g-Criterion:	46.48766
Residual Variance (Chi²/df):	0.02423
r²:	0.003
F:	0.090
p(F) (df: 1;28):	0.766

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0.100 data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No statistically significant concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significantly different from zero).
Due to the lacking concentration/response the shown ECx could not be valid.
Nonetheless, the user decided to show the ECx, confidence limits and graphs if possible.

Results of the probit analysis

Tab. 5: Results of the probit analysis with reproduction - shelled embryos at 28.0 d: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller's theorem).

Toxicity Metric	EC10	EC20	EC50
Value [ng/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 3.71845338079998E36 Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

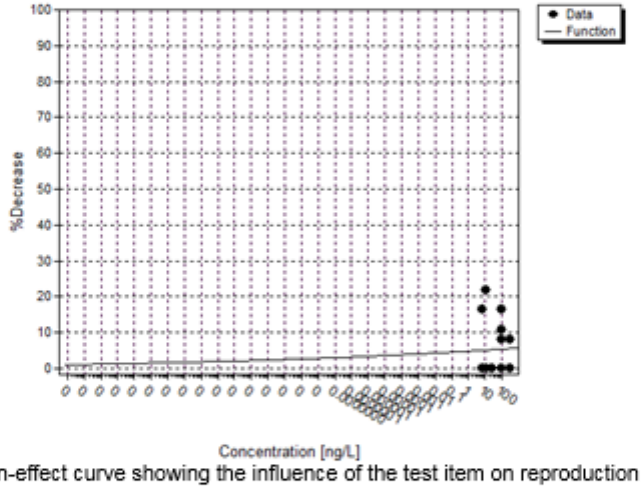


Fig. 2: Concentration-effect curve showing the influence of the test item on reproduction - shelled embryos of the introduced Potamopyrgus antipodarum as observed after 28.0 d

Threshold concentrations (NOEC) for Reproduction - shelled embryos at 28.0 d

Reproduction - shelled embryos [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Increase of reproduction - shelled embryos caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Increase
Control	3.58	0.763	6	
6.970	4.03	0.599	6	12.558
11.170	4.43	1.133	6	23.721
25.600	4.42	0.523	6	23.256
106.000	3.92	1.181	6	9.302
332.000	4.38	0.966	6	22.326

Statistical Characteristics of the Samples

Tab. 3: Statistical characteristics with reproduction - shelled embryos at 28.0 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [ng/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	3.58	3.45	2.70	4.70	6	0.763	21.3	0.311	8.7	2.78	4.38
6.970	4.03	4.25	3.00	4.70	6	0.599	14.8	0.244	6.1	3.40	4.66
11.170	4.43	4.50	2.80	5.70	6	1.133	25.5	0.462	10.4	3.24	5.62
25.600	4.42	4.35	3.80	5.20	6	0.523	11.8	0.214	4.8	3.87	4.97
106.000	3.92	3.25	3.00	5.80	6	1.181	30.1	0.482	12.3	2.68	5.16
332.000	4.38	4.25	3.30	6.00	6	0.966	22.0	0.394	9.0	3.37	5.40

Shapiro-Wilk´s Test on Normal Distribution

Tab. 4: Shapiro-Wilk´s Test on Normal Distribution with reproduction - shelled embryos at 28.0 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [ng/L]	Mean	s	n
Control	3.58	0.763	6
6.970	4.03	0.599	6
11.170	4.43	1.133	6
25.600	4.42	0.523	6
106.000	3.92	1.181	6
332.000	4.38	0.966	6

Results:

Number of residuals = 29; Shapiro-Wilk´s W = 0.981; p(W) = 0.868; p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 5: Levene´s Test on Variance Homogeneity (with Residuals) with reproduction - shelled embryos at 28.0 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	1.8242	5	0.3648	2.328	0.067
Residuals	4.7024	30	0.1567		
Total	6.527	35			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 6: Trend analysis by contrasts (monotonicity of concentration/response) with reproduction - shelled embryos at 28.0 d: Psi: sum of means weighted by contrasts; s(psi): standard error of psi; df: degrees of freedom; t: t-statistic; p(t): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	s(psi)	df	t	p(t)
Linear	3.6333	3.0627	30	1.186	0.122
Quadratic	-3.5167	3.3550	30	-1.048	0.152

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, thus the selected Williams test was replaced by Dunnett test.

Dunnett`s Multiple t-test Procedure

Tab. 7: Dunnett´s multiple t-test procedure with reproduction - shelled embryos at 28.0 d: Comparison of treatments with "Control". Significance was Alpha = 0.050, one-sided smaller (multiple level); Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [ng/L]	Mean	s	df	%MDD	t	t*	Sign.
Control	3.58	0.8967					
6.970	4.03	0.8967	30	-33.806	0.87	-2.34	-
11.170	4.43	0.8967	30	-33.806	1.64	-2.34	-
25.600	4.42	0.8967	30	-33.806	1.61	-2.34	-
106.000	3.92	0.8967	30	-33.806	0.64	-2.34	-
332.000	4.38	0.8967	30	-33.806	1.55	-2.34	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 332.000 ng/L.

B.1.5 Offspring number – unshelled embryos

Evaluation of a Metric Response: Snail Reproduction test with Dienogest

General:

Test identification/project no.	Snail Reproduction test with Dienogest
Test item	Dienogest
Unit of test item concentration	ng/L
Start of experiment on day	10/7/2019
Date and time of the evaluation	21/11/2019; 08:41:11
Raw data filename: data.xls	ToxRat Auswertung Reproduction_unshelled embryos_raw

Test design

Number of treatments (incl. control(s))	6
Duration of the test	28.0 d
Measurement variable	Reproduction - unshelled embryos
Test system	Potamopyrgus antipodarum

Reproduction - unshelled embryos of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Reproduction - unshelled embryos [embryo number] of Potamopyrgus antipodarum as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (from Tabs Reproduction - unshelled embryos)

Treatm. [ng/L]	Control	6.970	11.170	25.600	106.000	332.000
28.0 d	5.70	6.00	4.20	4.30	3.20	3.70
	4.00	5.20	4.80	4.20	4.50	3.30
	2.30	4.00	3.30	4.50	4.80	3.30
	3.20	3.00	6.20	3.30	4.30	5.00
	4.20	3.50	6.30	4.00	4.30	4.30
	2.50	4.20	4.30	3.50	6.00	5.00
Mean:	3.65	4.32	4.85	3.97	4.52	4.10
Std.Dev.:	1.263	1.107	1.188	0.472	0.906	0.787
n:	6	6	6	6	6	6
CV:	34.601	25.647	24.492	11.896	20.069	19.205

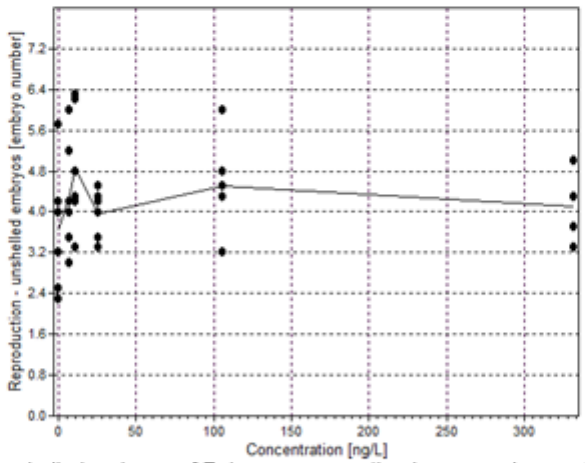


Fig. 1: Reproduction - unshelled embryos of Potamopyrgus antipodarum as observed under presence of the test item after 28.0 d.

Effective Concentrations (ECx) for Reproduction - unshelled embryos at 28.0 d

Reproduction - unshelled embryos [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of reproduction - unshelled embryos caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Decrease
Control	3.65	1.263	6	
6.970	4.32	1.107	6	-18.265
11.170	4.85	1.188	6	-32.877
25.600	3.97	0.472	6	-8.676
106.000	4.52	0.906	6	-23.744
332.000	4.10	0.787	6	-12.329

Probit analysis using linear max. likelihood regression

Tab. 3: Probit analysis using linear max. likelihood regression with reproduction - unshelled embryos at 28.0 d: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [ng/L]	Log(x)	%Decrease	n	Emp. Probit	Weight	Reg. Probit
6.970	0.843	0.00	1	-2.867	0.242	-1.191
6.970	0.843	0.00	1	-2.318	0.242	-1.191
6.970	0.843	0.00	1	-1.494	0.242	-1.191
6.970	0.843	17.81	1	-0.807	0.242	-1.191
6.970	0.843	4.11	1	-1.150	0.242	-1.191
6.970	0.843	0.00	1	-1.631	0.242	-1.191
11.170	1.048	0.00	1	-1.631	0.242	-1.190
11.170	1.048	0.00	1	-2.043	0.242	-1.190
11.170	1.048	9.59	1	-1.013	0.242	-1.190
11.170	1.048	0.00	1	-3.005	0.242	-1.190
11.170	1.048	0.00	1	-3.073	0.242	-1.190
11.170	1.048	0.00	1	-1.700	0.242	-1.190
25.600	1.408	0.00	1	-1.700	0.243	-1.190
25.600	1.408	0.00	1	-1.631	0.243	-1.190
25.600	1.408	0.00	1	-1.837	0.243	-1.190
25.600	1.408	9.59	1	-1.013	0.243	-1.190
25.600	1.408	0.00	1	-1.494	0.243	-1.190
25.600	1.408	4.11	1	-1.150	0.243	-1.190
106.000	2.025	12.33	1	-0.944	0.243	-1.189
106.000	2.025	0.00	1	-1.837	0.243	-1.189
106.000	2.025	0.00	1	-2.043	0.243	-1.189
106.000	2.025	0.00	1	-1.700	0.243	-1.189
106.000	2.025	0.00	1	-1.700	0.243	-1.189

106.000	2.025	0.00	1	-2.867	0.243	-1.189
332.000	2.521	0.00	1	-1.288	0.244	-1.188
332.000	2.521	9.59	1	-1.013	0.244	-1.188
332.000	2.521	9.59	1	-1.013	0.244	-1.188
332.000	2.521	0.00	1	-2.180	0.244	-1.188
332.000	2.521	0.00	1	-1.700	0.244	-1.188
332.000	2.521	0.00	1	-2.180	0.244	-1.188

excluded: value not in line with the chosen function

Inhibition greater than 100 or lower than 0% were replaced by 100.0% and 0%, respectively.

Parameters of the probit analysis

Tab. 4: Parameters of the probit analysis with reproduction - unshelled embryos at 28.0 d: Results of the regression analysis

Parameter	Value
Computation runs:	2
Slope b:	0.00430
Intercept a:	-1.66189
Variance of b:	0.35364
Goodness of Fit	
Chi²:	0.42380
Degrees of freedom:	28
p(Chi²):	1.000
Log EC50:	386.84906
SE Log EC50:	53,332.91307
g-Criterion:	1,216.94751
Residual Variance (Chi²/df):	0.01514
r²:	0.000
F:	0.003
p(F) (df: 1;28):	0.954

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0.100 data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No statistically significant concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significantly different from zero).
Due to the lacking concentration/response the shown ECx could not be valid.
Nonetheless, the user decided to show the ECx, confidence limits and graphs if possible.

Results of the probit analysis

Tab. 5: Results of the probit analysis with reproduction - unshelled embryos at 28.0 d: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	EC10	EC20	EC50
Value [ng/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 0.000 Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

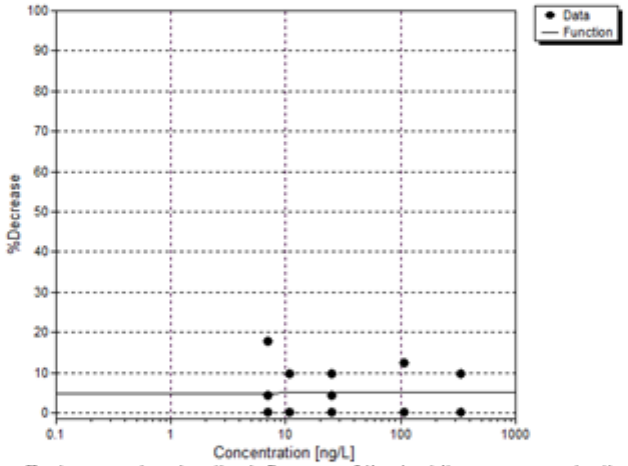


Fig. 2: Concentration-effect curve showing the influence of the test item on reproduction - unshelled embryos of the introduced Potamopyrgus antipodarum as observed after 28.0 d

Threshold concentrations (NOEC) for Reproduction - unshelled embryos at 28.0 d

Reproduction - unshelled embryos [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of reproduction - unshelled embryos caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Decrease
Control	3.65	1.263	6	
6.970	4.32	1.107	6	-18.265
11.170	4.85	1.188	6	-32.877
25.600	3.97	0.472	6	-8.676
106.000	4.52	0.906	6	-23.744
332.000	4.10	0.787	6	-12.329

Statistical Characteristics of the Samples

Tab. 3: Statistical characteristics with reproduction - unshelled embryos at 28.0 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [ng/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	3.65	3.60	2.30	5.70	6	1.263	34.6	0.516	14.1	2.32	4.98
6.970	4.32	4.10	3.00	6.00	6	1.107	25.6	0.452	10.5	3.15	5.48
11.170	4.85	4.55	3.30	6.30	6	1.188	24.5	0.485	10.0	3.60	6.10
25.600	3.97	4.10	3.30	4.50	6	0.472	11.9	0.193	4.9	3.47	4.46
106.000	4.52	4.40	3.20	6.00	6	0.906	20.1	0.370	8.2	3.57	5.47
332.000	4.10	4.00	3.30	5.00	6	0.787	19.2	0.321	7.8	3.27	4.93

Shapiro-Wilk´s Test on Normal Distribution

Tab. 4: Shapiro-Wilk´s Test on Normal Distribution with reproduction - unshelled embryos at 28.0 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [ng/L]	Mean	s	n
Control	3.65	1.263	6
6.970	4.32	1.107	6
11.170	4.85	1.188	6
25.600	3.97	0.472	6
106.000	4.52	0.906	6
332.000	4.10	0.787	6

Results:

Number of residuals = 32; Shapiro-Wilk´s W = 0.973; p(W) = 0.595; p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 5: Levene´s Test on Variance Homogeneity (with Residuals) with reproduction - unshelled embryos at 28.0 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	1.6150	5	0.3230	1.146	0.358
Residuals	8.4561	30	0.2819		
Total	10.071	35			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 6: Trend analysis by contrasts (monotonicity of concentration/response) with reproduction - unshelled embryos at 28.0 d: Psi: sum of means weighted by contrasts; s(psi): standard error of psi; df: degrees of freedom; t: t-statistic; p(t): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	s(psi)	df	t	p(t)
Linear	-1.9667	3.3859	30	-0.581	0.283
Quadratic	5.3500	3.7091	30	1.442	0.080

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, thus the selected Williams test was replaced by Dunnett test.

Dunnett`s Multiple t-test Procedure

Tab. 7: Dunnett´s multiple t-test procedure with reproduction - unshelled embryos at 28.0 d: Comparison of treatments with "Control". Significance was Alpha = 0.050, one-sided smaller (multiple level); Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [ng/L]	Mean	s	df	%MDD	t	t*	Sign.
Control	3.65	0.9913					
6.970	4.32	0.9913	30	-36.692	1.16	-2.34	-
11.170	4.85	0.9913	30	-36.692	2.10	-2.34	-
25.600	3.97	0.9913	30	-36.692	0.55	-2.34	-
106.000	4.52	0.9913	30	-36.692	1.51	-2.34	-
332.000	4.10	0.9913	30	-36.692	0.79	-2.34	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 332.000 ng/L.

B.2 Statistical evaluations – Reproduction test with Dexamethasone

All statistical calculations were based on geometric mean measured concentrations of Dexamethasone. All results and explanations are directly imported from ToxRat (6).

B.2.1 Survival

Statistical Evaluation of a Quantal Response: Snail Reproduction test with Dexamethasone

General:	
Test identification/project no.	Snail Reproduction test with Dexamethasone
Test item	Dexamethasone
Unit of test item concentration	µg/L
Start of experiment on day	2/10/2019
Date and time of the evaluation	21/11/2019; 08:51:28
Raw data filename:	ToxRat Auswertung Mortality_raw data.xls
Test design	
Number of treatments (incl. control(s))	6
Duration of the test	28 d
Measurement variable	Mortality
Test system	Potamopyrgus antipodarum

Mortality of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Mortality of Potamopyrgus antipodarum as dependent on concentration of the test item and time (from InputRawData)

Treatm. [µg/L]	Control	1.060	3.540	10.500	36.300	105.000
0 d	6	6	6	6	6	6
	6	6	6	6	6	6
	6	6	6	6	6	6
	6	6	6	6	6	6
	6	6	6	6	6	6
	6	6	6	6	6	6
Total Introduced	36	36	36	36	36	36
n:	6	6	6	6	6	6
28 d	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
Total Dead:	0	0	0	0	0	0
n:	6	6	6	6	6	6

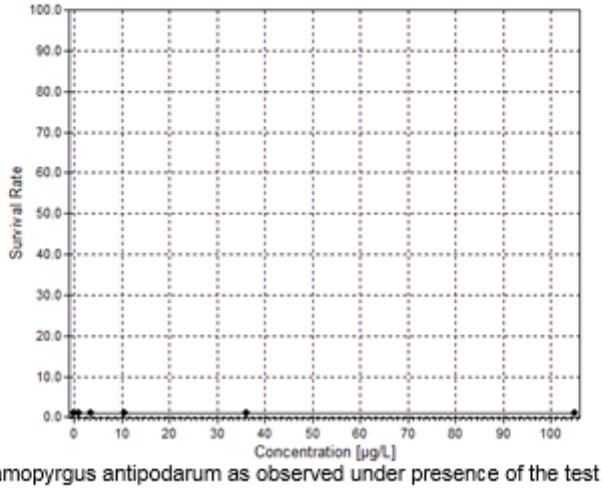


Fig. 1: Mortality of Potamopyrgus antipodarum as observed under presence of the test item after 28 d.

Lethal Concentrations (LCx) for Mortality at 28 d

Overview Mortality

Tab. 2: Overview Mortality: Overview over the effects on mortality in Potamopyrgus antipodarum at 28 d

Treatm.[µg/L]	Total Introduced	Survived	Dead	% Mortality
Control	36	36	0	0.000
1.060	36	36	0	0.000
3.540	36	36	0	0.000
10.500	36	36	0	0.000
36.300	36	36	0	0.000
105.000	36	36	0	0.000

Because no change in mortality was to be observed, no further computations have been performed for 28 d.

Because no change in mortality was to be observed, no further computations have been performed for 28 d.

Threshold concentrations (NOEC) for Mortality at 28 d

To justify the use of the Step-down Cochran-Armitage test at first a trend analysis by contrasts using proportions was performed.

Qualitative Trend Analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 2: Qualitative trend analysis by contrasts (monotonicity of concentration/response) with mortality at 28 d: Psi: total of proportions weighted by contrasts; Var(psi): variance of psi; df: degrees of freedom; Chi²: Chi²-statistic; p(Chi²): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	Var(psi)	df	Chi²	p(Chi²)
Linear	0.0000	0.0000	5	NAN	1.000
Quadratic	0.0000	0.0000	5	NAN	1.000

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, nonetheless the user-selected Step-down Cochran-Armitage test was performed.

Ahead of the Cochran-Armitage test Tarone´s test had to be performed to test for extra-binomial variance.

Tarone´s Test Procedure

Tab. 3: Tarone Test with mortality at 28 d: Treatment-wise testing the homgeneity of proportions (Alpha = 0.010). The statistic TZ has an asymptotic chi² distribution with one degree of freedom and measures the deviation from homogeneity. Ho (Phi = 0; i.e. homogeneity) is accepted, if the probability p(TZ) > Alpha; p(TZ) is the probability that the deviation from homogeneity observed in the treatment(s) is due to chance.

Treatm.[µg/L]	Introduced	Survived	Dead	TZp(TZ)	sign.
Control	36	36	0	3.6000.058	-
1.060	36	36	0	3.6000.058	-
3.540	36	36	0	3.6000.058	-
10.500	36	36	0	3.6000.058	-
36.300	36	36	0	3.6000.058	-
105.000	36	36	0	3.6000.058	-

+: significant; -: non-significant

In treatments no signs of extra-bionmial variance were found.

Step-down Cochran-Armitage Test Procedure

Tab. 4: Step-down Cochran-Armitage Test Procedure with mortality at 28 d: Step-down test to detect an increasing trend in responses (Alpha is 0.050; one-sided greater); Chi²(tot): total (Pearson) Chi²; z(trend): standardized one-sided deviation due to the linear upward trend; Chi²(err): unexplained component of Chi²(tot); p(tot|trend|err): probabilities that the observed results could be due to chance; Ho (no trend) is accepted, if p(trend) > Alpha. Note that the step-down test terminates after the first non-significant treatment is encountered

Treatm. [µg/L]	Total	Introduced	Dead%	Mortality	Chi²(tot)	p(tot)	Chi²(err)	p(err)	|z|(trend)	p(trend)	Sign.
Control	36	0	0.000								
1.060	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000		-
3.540	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000		-
10.500	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000		-
36.300	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000		-
105.000	36	0	0.000	0.000	1.000	0.000	<0.001	0.000	1.000		-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 105.000 µg/L.

B.2.2 Length

Evaluation of a Metric Response: Snail Reproduction test with Dexamethasone

General:	
Test identification/project no.	Snail Reproduction test with Dexamethasone
Test item	Dexamethasone
Unit of test item concentration	µg/L
Start of experiment on day	2/10/2019
Date and time of the evaluation	21/11/2019; 08:47:54
Raw data filename:	ToxRat Auswertung Length_raw data.xls
Test design	
Number of treatments (incl. control(s))	6
Duration of the test	28.0 d
Measurement variable	Length at test end
Test system	Potamopyrgus antipodarum

Length at test end of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Length at test end [mm] of Potamopyrgus antipodarum as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (from Tabs Length at test end)

Treatm. [µg/L]	Control	1.060	3.540	10.500	36.300	105.000
28.0 d	3.78	3.65	3.78	3.81	3.79	3.64
	3.73	3.73	3.66	3.80	3.64	3.75
	3.98	3.66	3.64	3.67	3.77	3.61
	3.65	3.72	3.62	3.63	3.83	3.61
	3.76	3.71	3.60	3.70	3.91	3.77
	3.65	3.68	3.72	3.75	3.72	3.79
Mean:	3.76	3.69	3.67	3.73	3.78	3.69
Std.Dev.:	0.122	0.033	0.068	0.072	0.092	0.084
n:	6	6	6	6	6	6
CV:	3.234	0.897	1.848	1.940	2.448	2.269

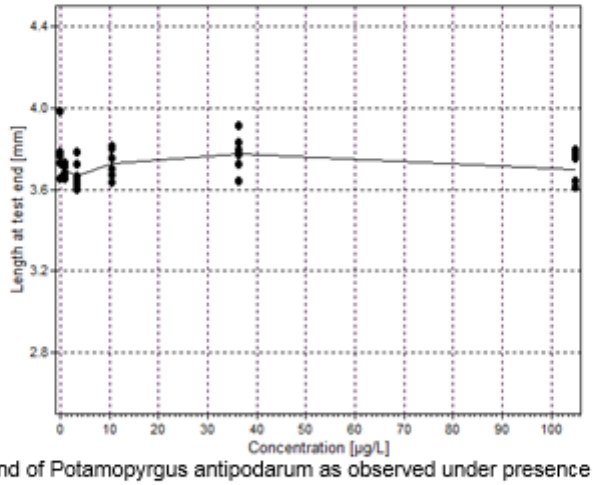


Fig. 1: Length at test end of Potamopyrgus antipodarum as observed under presence of the test item after 28.0 d.

Effective Concentrations (ECx) for Length at test end at 28.0 d

Length at test end [mm] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of length at test end caused by the test item after 28.0 d.

Treatm.[µg/L]	Mean	Std. Dev.	n	%Decrease
Control	3.76	0.122	6	
1.060	3.69	0.033	6	1.774
3.540	3.67	0.068	6	2.350
10.500	3.73	0.072	6	0.843
36.300	3.78	0.092	6	-0.488
105.000	3.69	0.084	6	1.685

Probit analysis using linear max. likelihood regression

Tab. 3: Probit analysis using linear max. likelihood regression with length at test end at 28.0 d: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [µg/L]	Log(x)	%Decrease	n	Emp. Probit	Weight	Reg. Probit
1.060	0.025	2.88	1	-1.181	0.234	-1.205
1.060	0.025	0.75	1	-1.234	0.234	-1.205
1.060	0.025	2.62	1	-1.188	0.234	-1.205
1.060	0.025	1.02	1	-1.228	0.234	-1.205
1.060	0.025	1.29	1	-1.221	0.234	-1.205
1.060	0.025	2.08	1	-1.201	0.234	-1.205
3.540	0.549	0.00	1	-1.268	0.232	-1.209
3.540	0.549	2.62	1	-1.188	0.232	-1.209
3.540	0.549	3.15	1	-1.174	0.232	-1.209
3.540	0.549	3.68	1	-1.161	0.232	-1.209
3.540	0.549	4.21	1	-1.148	0.232	-1.209
3.540	0.549	1.02	1	-1.228	0.232	-1.209
10.500	1.021	0.00	1	-1.288	0.230	-1.213
10.500	1.021	0.00	1	-1.281	0.230	-1.213
10.500	1.021	2.35	1	-1.194	0.230	-1.213
10.500	1.021	3.41	1	-1.168	0.230	-1.213
10.500	1.021	1.55	1	-1.214	0.230	-1.213
10.500	1.021	0.22	1	-1.248	0.230	-1.213
36.300	1.560	0.00	1	-1.274	0.227	-1.217
36.300	1.560	3.15	1	-1.174	0.227	-1.217
36.300	1.560	0.00	1	-1.261	0.227	-1.217
36.300	1.560	0.00	1	-1.301	0.227	-1.217
36.300	1.560	0.00	1	-1.354	0.227	-1.217
36.300	1.560	1.02	1	-1.228	0.227	-1.217

105.000	2.021	3.15	1	-1.174	0.225	-1.221
105.000	2.021	0.22	1	-1.248	0.225	-1.221
105.000	2.021	3.95	1	-1.154	0.225	-1.221
105.000	2.021	3.95	1	-1.154	0.225	-1.221
105.000	2.021	0.00	1	-1.261	0.225	-1.221
105.000	2.021	0.00	1	-1.274	0.225	-1.221

excluded: value not in line with the chosen function

Inhibition greater than 100 or lower than 0% were replaced by 100.0% and 0%, respectively.

Parameters of the probit analysis

Tab. 4: Parameters of the probit analysis with length at test end at 28.0 d: Results of the regression analysis

Parameter	Value
Computation runs:	2
Slope b:	-0.02121
Intercept a:	-1.69583
Variance of b:	0.28990
Goodness of Fit	
Chi²:	0.04043
Degrees of freedom:	28
p(Chi²):	1.000
Log EC50:	-79.94791
SE Log EC50:	2,055.48437
g-Criterion:	3.90344
Residual Variance (Chi²/df):	0.00144
r²:	0.037
F:	1.075
p(F) (df: 1;28):	0.309

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0.100 data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No statistically significant concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significantly different from zero).
Due to the lacking concentration/response the shown ECx could not be valid.
Nonetheless, the user decided to show the ECx, confidence limits and graphs if possible.

Statistical Results on the Length at test end of Potamopyrgus antipodarum

Results of the probit analysis

Tab. 5: Results of the probit analysis with length at test end at 28.0 d: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller's theorem).

Toxicity Metric	EC10	EC20	EC50
Value [µg/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 0.000 Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

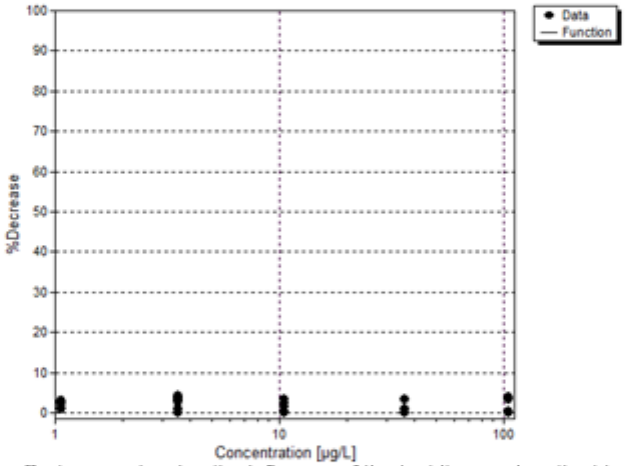


Fig. 2: Concentration-effect curve showing the influence of the test item on length at test end of the introduced Potamopyrgus antipodarum as observed after 28.0 d

Threshold concentrations (NOEC) for Length at test end at 28.0 d

Length at test end [mm] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of length at test end caused by the test item after 28.0 d.

Treatm.[µg/L]	Mean	Std. Dev.	n	%Decrease
Control	3.76	0.122	6	
1.060	3.69	0.033	6	1.774
3.540	3.67	0.068	6	2.350
10.500	3.73	0.072	6	0.843
36.300	3.78	0.092	6	-0.488
105.000	3.69	0.084	6	1.685

Statistical Characteristics of the Samples

Tab. 3: Statistical characteristics with length at test end at 28.0 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	3.76	3.75	3.65	3.98	6	0.122	3.2	0.050	1.3	3.63	3.89
1.060	3.69	3.70	3.65	3.73	6	0.033	0.9	0.014	0.4	3.66	3.73
3.540	3.67	3.65	3.60	3.78	6	0.068	1.8	0.028	0.8	3.60	3.74
10.500	3.73	3.73	3.63	3.81	6	0.072	1.9	0.030	0.8	3.65	3.80
36.300	3.78	3.78	3.64	3.91	6	0.092	2.4	0.038	1.0	3.68	3.87
105.000	3.69	3.70	3.61	3.79	6	0.084	2.3	0.034	0.9	3.61	3.78

Shapiro-Wilk´s Test on Normal Distribution

Tab. 4: Shapiro-Wilk´s Test on Normal Distribution with length at test end at 28.0 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	3.76	0.122	6
1.060	3.69	0.033	6
3.540	3.67	0.068	6
10.500	3.73	0.072	6
36.300	3.78	0.092	6
105.000	3.69	0.084	6

Results:

Number of residuals = 33; Shapiro-Wilk´s W = 0.982; p(W) = 0.836; p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Statistical Results on the Length at test end of Potamopyrgus antipodarum

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 5: Levene´s Test on Variance Homogeneity (with Residuals) with length at test end at 28.0 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	0.0107	5	0.0021	1.027	0.420
Residuals	0.0625	30	0.0021		
Total	0.073	35			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 6: Trend analysis by contrasts (monotonicity of concentration/response) with length at test end at 28.0 d: Psi: sum of means weighted by contrasts; s(psi): standard error of psi; df: degrees of freedom; t: t-statistic; p(t): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	s(psi)	df	t	p(t)
Linear	0.0050	0.2833	30	0.018	0.493
Quadratic	-0.2117	0.3103	30	-0.682	0.250

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, thus the selected Williams test was replaced by Dunnett test.

Dunnett`s Multiple t-test Procedure

Tab. 7: Dunnett´s multiple t-test procedure with length at test end at 28.0 d: Comparison of treatments with "Control". Significance was Alpha = 0.050, one-sided smaller (multiple level); Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	%MDD	t	t*	Sign.
Control	3.76	0.0829					
1.060	3.69	0.0829	30	-2.982	-1.39	-2.34	-
3.540	3.67	0.0829	30	-2.982	-1.84	-2.34	-
10.500	3.73	0.0829	30	-2.982	-0.66	-2.34	-
36.300	3.78	0.0829	30	-2.982	0.38	-2.34	-
105.000	3.69	0.0829	30	-2.982	-1.32	-2.34	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 105.000 µg/L.

B.2.3 Offspring number – Total embryos

Evaluation of a Metric Response: Snail Reproduction test with Dexamethasone

General:

Test identification/project no.	Snail Reproduction test with Dexamethasone
Test item	Dexamethasone
Unit of test item concentration	µg/L
Start of experiment on day	2/10/2019
Date and time of the evaluation	21/11/2019; 08:59:43
Raw data filename:	ToxRat Auswertung Reproduction_total embryos_raw data.xls

Test design

Number of treatments (incl. control(s))	6
Duration of the test	28.0 d
Measurement variable	Reproduction
Test system	Potamopyrgus antipodarum

Reproduction of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Reproduction [embryo number] of Potamopyrgus antipodarum as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (from Tabs Reproduction)

Treatm. [µg/L]	Control	1.060	3.540	10.500	36.300	105.000
28.0 d	16.20	13.50	13.80	14.50	15.00	11.80
	17.30	15.50	11.70	16.50	14.80	12.30
	16.50	12.80	10.00	15.50	12.80	14.70
	12.30	15.00	14.00	14.70	16.30	11.70
	15.30	13.50	14.20	18.00	16.70	11.00
	13.00	14.20	10.70	15.70	15.70	15.80
Mean:	15.10	14.25	12.40	15.82	15.22	12.88
Std.Dev.:	2.015	1.253	1.838	1.291	1.391	1.911
n:	6	6	6	6	6	6
CV:	13.344	8.796	14.826	8.160	9.138	14.837

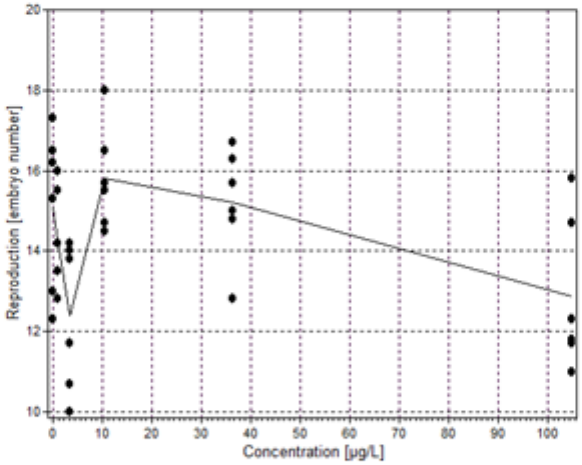


Fig. 1: Reproduction of Potamopyrgus antipodarum as observed under presence of the test item after 28.0 d.

Effective Concentrations (ECx) for Reproduction at 28.0 d

Reproduction [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of reproduction caused by the test item after 28.0 d.

Treatm.[µg/L]	Mean	Std. Dev.	n	%Decrease
Control	15.10	2.015	6	
1.060	14.25	1.253	6	5.629
3.540	12.40	1.838	6	17.881
10.500	15.82	1.291	6	-4.746
36.300	15.22	1.391	6	-0.773
105.000	12.88	1.911	6	14.680

Probit analysis using linear max. likelihood regression

Tab. 3: Probit analysis using linear max. likelihood regression with reproduction at 28.0 d: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [µg/L]	Log(x)	%Decrease	n	Emp. Probit	Weight	Reg. Probit
1.060	0.025	10.60	1	-0.988	0.150	-1.376
1.060	0.025	0.00	1	-1.320	0.150	-1.376
1.060	0.025	15.23	1	-0.872	0.150	-1.376
1.060	0.025	0.00	1	-1.403	0.150	-1.376
1.060	0.025	10.60	1	-0.988	0.150	-1.376
1.060	0.025	5.96	1	-1.104	0.150	-1.376
3.540	0.549	8.61	1	-1.038	0.157	-1.361
3.540	0.549	22.52	1	-0.689	0.157	-1.361
3.540	0.549	33.77	1	-0.407	0.157	-1.361
3.540	0.549	7.28	1	-1.071	0.157	-1.361
3.540	0.549	5.96	1	-1.104	0.157	-1.361
3.540	0.549	29.14	1	-0.523	0.157	-1.361
10.500	1.021	3.97	1	-1.154	0.163	-1.348
10.500	1.021	0.00	1	-1.486	0.163	-1.348
10.500	1.021	0.00	1	-1.320	0.163	-1.348
10.500	1.021	2.65	1	-1.187	0.163	-1.348
10.500	1.021	0.00	1	-1.735	0.163	-1.348
10.500	1.021	0.00	1	-1.353	0.163	-1.348
36.300	1.560	0.66	1	-1.237	0.169	-1.333
36.300	1.560	1.99	1	-1.204	0.169	-1.333
36.300	1.560	15.23	1	-0.872	0.169	-1.333
36.300	1.560	0.00	1	-1.453	0.169	-1.333
36.300	1.560	0.00	1	-1.519	0.169	-1.333
36.300	1.560	0.00	1	-1.353	0.169	-1.333

105.000	2.021	21.85	1	-0.706	0.175	-1.319
105.000	2.021	18.54	1	-0.789	0.175	-1.319
105.000	2.021	2.65	1	-1.187	0.175	-1.319
105.000	2.021	22.52	1	-0.689	0.175	-1.319
105.000	2.021	27.15	1	-0.573	0.175	-1.319
105.000	2.021	0.00	1	-1.370	0.175	-1.319

excluded: value not in line with the chosen function

Inhibition greater than 100 or lower than 0% were replaced by 100.0% and 0%, respectively.

Parameters of the probit analysis

Tab. 4: Parameters of the probit analysis with reproduction at 28.0 d: Results of the regression analysis

Parameter	Value
Computation runs:	8
Slope b:	0.02843
Intercept a:	-1.37695
Variance of b:	0.41009
Goodness of Fit	
Chi²:	1.95195
Degrees of freedom:	28
p(Chi²):	1.000
Log EC50:	48.43378
SE Log EC50:	1,066.91594
g-Criterion:	148.41606
Residual Variance (Chi²/df):	0.06971
r²:	0.001
F:	0.028
p(F) (df: 1;28):	0.868

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0.100 data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance). The coefficient of determination, r² (0 <= r² <= 1), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No statistically significant concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significantly different from zero).
Due to the lacking concentration/response the shown ECx could not be valid.
Nonetheless, the user decided to show the ECx, confidence limits and graphs if possible.

Results of the probit analysis

Tab. 5: Results of the probit analysis with reproduction at 28.0 d: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	EC10	EC20	EC50
Value [µg/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 1.49516130038736E35 Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

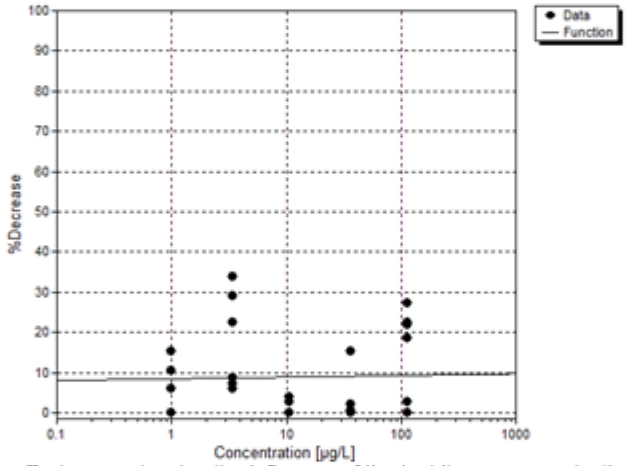


Fig. 2: Concentration-effect curve showing the influence of the test item on reproduction of the introduced Potamopyrgus antipodarum as observed after 28.0 d

Threshold concentrations (NOEC) for Reproduction at 28.0 d

Reproduction [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of reproduction caused by the test item after 28.0 d.

Treatm.[µg/L]	Mean	Std. Dev.	n	%Decrease
Control	15.10	2.015	6	
1.060	14.25	1.253	6	5.629
3.540	12.40	1.838	6	17.881
10.500	15.82	1.291	6	-4.746
36.300	15.22	1.391	6	-0.773
105.000	12.88	1.911	6	14.680

Statistical Characteristics of the Samples

Tab. 3: Statistical characteristics with reproduction at 28.0 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	15.10	15.75	12.30	17.30	6	2.015	13.3	0.823	5.4	12.99	17.21
1.060	14.25	13.85	12.80	16.00	6	1.253	8.8	0.512	3.6	12.93	15.57
3.540	12.40	12.75	10.00	14.20	6	1.838	14.8	0.751	6.1	10.47	14.33
10.500	15.82	15.60	14.50	18.00	6	1.291	8.2	0.527	3.3	14.46	17.17
36.300	15.22	15.35	12.80	16.70	6	1.391	9.1	0.568	3.7	13.76	16.68
105.000	12.88	12.05	11.00	15.80	6	1.911	14.8	0.780	6.1	10.88	14.89

Shapiro-Wilk´s Test on Normal Distribution

Tab. 4: Shapiro-Wilk´s Test on Normal Distribution with reproduction at 28.0 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	15.10	2.015	6
1.060	14.25	1.253	6
3.540	12.40	1.838	6
10.500	15.82	1.291	6
36.300	15.22	1.391	6
105.000	12.88	1.911	6

Results:

Number of residuals = 34; Shapiro-Wilk´s W = 0.967; p(W) = 0.383; p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 5: Levene´s Test on Variance Homogeneity (with Residuals) with reproduction at 28.0 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	3.4029	5	0.6806	1.178	0.343
Residuals	17.3368	30	0.5779		
Total	20.740	35			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 6: Trend analysis by contrasts (monotonicity of concentration/response) with reproduction at 28.0 d: Psi: sum of means weighted by contrasts; s(psi): standard error of psi; df: degrees of freedom; t: t-statistic; p(t): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	s(psi)	df	t	p(t)
Linear	4.7667	5.6236	30	0.848	0.202
Quadratic	2.4167	6.1603	30	0.392	0.349

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, thus the selected Williams test was replaced by Dunnett test.

Dunnett`s Multiple t-test Procedure

Tab. 7: Dunnett´s multiple t-test procedure with reproduction at 28.0 d: Comparison of treatments with "Control". Significance was Alpha = 0.050, one-sided smaller (multiple level); Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	%MDD	t	t*	Sign.
Control	15.10	1.6464					
1.060	14.25	1.6464	30	-14.730	-0.89	-2.34	-
3.540	12.40	1.6464	30	-14.730	-2.84	-2.34	+
10.500	15.82	1.6464	30	-14.730	0.75	-2.34	-
36.300	15.22	1.6464	30	-14.730	0.12	-2.34	-
105.000	12.88	1.6464	30	-14.730	-2.33	-2.34	-

+: significant; -: non-significant

The NOEC cannot be determined by the program (expert judgement required).

B.2.4 Offspring number – shelled embryos

Evaluation of a Metric Response: Snail Reproduction test with Dexamethasone

General:

Test identification/project no.	Snail Reproduction test with Dexamethasone
Test item	Dexamethasone
Unit of test item concentration	µg/L
Start of experiment on day	2/10/2019
Date and time of the evaluation	21/11/2019; 08:54:58
Raw data filename: data.xls	ToxRat Auswertung Reproduction_shelled embryos_raw

Test design

Number of treatments (incl. control(s))	6
Duration of the test	28.0 d
Measurement variable	Reproduction - shelled embryos
Test system	Potamopyrgus antipodarum

Reproduction - shelled embryos of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Reproduction - shelled embryos [embryo number] of Potamopyrgus antipodarum as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (from Tabs Reproduction - shelled embryos)

Treatm. [µg/L]	Control	1.060	3.540	10.500	36.300	105.000
28.0 d	8.70	5.50	6.00	5.80	6.30	6.20
	10.80	7.20	6.30	8.50	7.80	5.50
	9.30	6.70	5.00	8.20	4.50	7.20
	8.00	8.00	6.30	7.20	7.80	4.20
	9.20	6.70	6.20	9.70	8.00	4.00
	5.30	4.70	6.20	7.70	8.00	7.70
Mean:	8.55	6.47	6.00	7.85	7.07	5.80
Std.Dev.:	1.840	1.188	0.502	1.313	1.414	1.524
n:	6	6	6	6	6	6
CV:	21.525	18.367	8.367	16.721	20.006	26.284

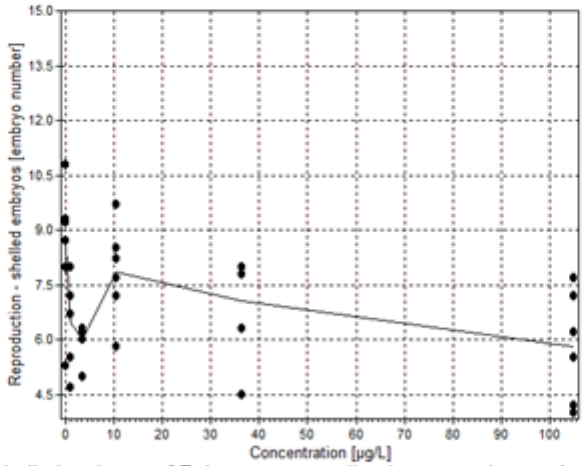


Fig. 1: Reproduction - shelled embryos of Potamopyrgus antipodarum as observed under presence of the test item after 28.0 d.

Effective Concentrations (ECx) for Reproduction - shelled embryos at 28.0 d

Reproduction - shelled embryos [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of reproduction - shelled embryos caused by the test item after 28.0 d.

Treatm.[µg/L]	Mean	Std. Dev.	n	%Decrease
Control	8.55	1.840	6	
1.060	6.47	1.188	6	24.366
3.540	6.00	0.502	6	29.825
10.500	7.85	1.313	6	8.187
36.300	7.07	1.414	6	17.349
105.000	5.80	1.524	6	32.164

Probit analysis using linear max. likelihood regression

Tab. 3: Probit analysis using linear max. likelihood regression with reproduction - shelled embryos at 28.0 d: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [µg/L]	Log(x)	%Decrease	n	Emp. Probit	Weight	Reg. Probit
1.060	0.025	35.67	1	-0.359	0.555	-0.768
1.060	0.025	15.79	1	-0.858	0.555	-0.768
1.060	0.025	21.64	1	-0.711	0.555	-0.768
1.060	0.025	6.43	1	-1.092	0.555	-0.768
1.060	0.025	21.64	1	-0.711	0.555	-0.768
1.060	0.025	45.03	1	-0.125	0.555	-0.768
3.540	0.549	29.82	1	-0.506	0.565	-0.756
3.540	0.549	26.32	1	-0.594	0.565	-0.756
3.540	0.549	41.52	1	-0.213	0.565	-0.756
3.540	0.549	26.32	1	-0.594	0.565	-0.756
3.540	0.549	27.49	1	-0.564	0.565	-0.756
3.540	0.549	27.49	1	-0.564	0.565	-0.756
10.500	1.021	32.16	1	-0.447	0.574	-0.745
10.500	1.021	0.58	1	-1.239	0.574	-0.745
10.500	1.021	4.09	1	-1.151	0.574	-0.745
10.500	1.021	15.79	1	-0.858	0.574	-0.745
10.500	1.021	0.00	1	-1.590	0.574	-0.745
10.500	1.021	9.94	1	-1.004	0.574	-0.745
36.300	1.560	26.32	1	-0.594	0.585	-0.733
36.300	1.560	8.77	1	-1.033	0.585	-0.733
36.300	1.560	47.37	1	-0.066	0.585	-0.733
36.300	1.560	8.77	1	-1.033	0.585	-0.733
36.300	1.560	6.43	1	-1.092	0.585	-0.733
36.300	1.560	6.43	1	-1.092	0.585	-0.733

105.000	2.021	27.49	1	-0.564	0.594	-0.722
105.000	2.021	35.67	1	-0.359	0.594	-0.722
105.000	2.021	15.79	1	-0.858	0.594	-0.722
105.000	2.021	50.88	1	0.022	0.594	-0.722
105.000	2.021	53.22	1	0.081	0.594	-0.722
105.000	2.021	9.94	1	-1.004	0.594	-0.722

excluded: value not in line with the chosen function

Inhibition greater than 100 or lower than 0% were replaced by 100.0% and 0%, respectively.

Parameters of the probit analysis

Tab. 4: Parameters of the probit analysis with reproduction - shelled embryos at 28.0 d: Results of the regression analysis

Parameter	Value
Computation runs:	6
Slope b:	0.02289
Intercept a:	-0.76839
Variance of b:	0.11603
Goodness of Fit	
Chi²:	4.25243
Degrees of freedom:	28
p(Chi²):	1.000
Log EC50:	33.57545
SE Log EC50:	484.18372
g-Criterion:	141.17041
Residual Variance (Chi²/df):	0.15187
r²:	0.001
F:	0.030
p(F) (df: 1;28):	0.864

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0.100 data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No statistically significant concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significantly different from zero).
Due to the lacking concentration/response the shown ECx could not be valid.
Nonetheless, the user decided to show the ECx, confidence limits and graphs if possible.

Results of the probit analysis

Tab. 5: Results of the probit analysis with reproduction - shelled embryos at 28.0 d: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	EC10	EC20	EC50
Value [µg/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 0.000 Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

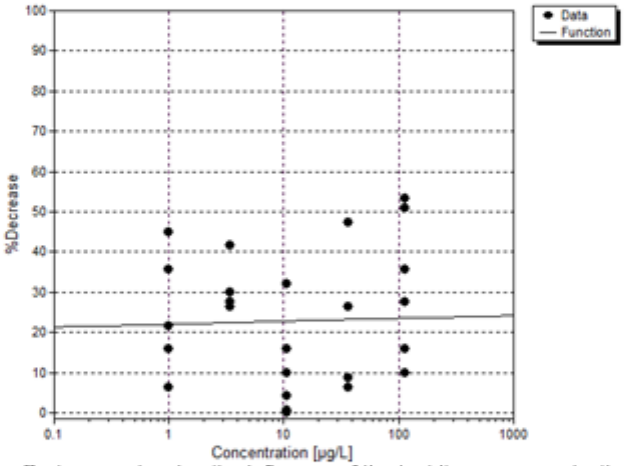


Fig. 2: Concentration-effect curve showing the influence of the test item on reproduction - shelled embryos of the introduced Potamopyrgus antipodarum as observed after 28.0 d

Threshold concentrations (NOEC) for Reproduction - shelled embryos at 28.0 d

Reproduction - shelled embryos [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of reproduction - shelled embryos caused by the test item after 28.0 d.

Treatm.[µg/L]	Mean	Std. Dev.	n	%Decrease
Control	8.55	1.840	6	
1.060	6.47	1.188	6	24.366
3.540	6.00	0.502	6	29.825
10.500	7.85	1.313	6	8.187
36.300	7.07	1.414	6	17.349
105.000	5.80	1.524	6	32.164

Statistical Characteristics of the Samples

Tab. 3: Statistical characteristics with reproduction - shelled embryos at 28.0 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	8.55	8.95	5.30	10.80	6	1.840	21.5	0.751	8.8	6.62	10.48
1.060	6.47	6.70	4.70	8.00	6	1.188	18.4	0.485	7.5	5.22	7.71
3.540	6.00	6.20	5.00	6.30	6	0.502	8.4	0.205	3.4	5.47	6.53
10.500	7.85	7.95	5.80	9.70	6	1.313	16.7	0.536	6.8	6.47	9.23
36.300	7.07	7.80	4.50	8.00	6	1.414	20.0	0.577	8.2	5.58	8.55
105.000	5.80	5.85	4.00	7.70	6	1.524	26.3	0.622	10.7	4.20	7.40

Shapiro-Wilk´s Test on Normal Distribution

Tab. 4: Shapiro-Wilk´s Test on Normal Distribution with reproduction - shelled embryos at 28.0 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	8.55	1.840	6
1.060	6.47	1.188	6
3.540	6.00	0.502	6
10.500	7.85	1.313	6
36.300	7.07	1.414	6
105.000	5.80	1.524	6

Results:

Number of residuals = 29; Shapiro-Wilk´s W = 0.978; p(W) = 0.788; p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 5: Levene´s Test on Variance Homogeneity (with Residuals) with reproduction - shelled embryos at 28.0 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	3.5187	5	0.7037	1.157	0.353
Residuals	18.2535	30	0.6085		
Total	21.772	35			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 6: Trend analysis by contrasts (monotonicity of concentration/response) with reproduction - shelled embryos at 28.0 d: Psi: sum of means weighted by contrasts; s(psi): standard error of psi; df: degrees of freedom; t: t-statistic; p(t): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	s(psi)	df	t	p(t)
Linear	10.1000	4.6448	30	2.174	0.019
Quadratic	-2.8167	5.0881	30	-0.554	0.292

The linear trend is significant (p <= 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts revealed a linear trend, thus the selected Williams test was performed.

Williams Multiple Sequential t-test Procedure

Tab. 7: Comparison of treatments with "Control" by the t test procedure after Williams with reproduction - shelled embryos at 28.0 d: Significance was Alpha = 0.050, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; 't*': critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments). Note that the step-down test terminates after the first non-significant treatment is encountered

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	8.55	1.3599						
1.060	6.47	1.3599	30	6.85	-15.583	-2.17	-1.70	+
3.540	6.00	1.3599	30	6.85	-16.308	-2.17	-1.78	+
10.500	7.85	1.3599	30	6.85	-16.538	-2.17	-1.80	+
36.300	7.07	1.3599	30	6.85	-16.657	-2.17	-1.81	+
105.000	5.80	1.3599	30	5.80	-16.722	-3.50	-1.82	+

+: significant; -: non-significant

The NOEC is lower than 1.060 µg/L.

B.2.5 Offspring number – unshelled embryos

Evaluation of a Metric Response: Snail Reproduction test with Dexamethasone

General:

Test identification/project no.	Snail Reproduction test with Dexamethasone
Test item	Dexamethasone
Unit of test item concentration	µg/L
Start of experiment on day	2/10/2019
Date and time of the evaluation	21/11/2019; 09:03:18
Raw data filename: data.xls	ToxRat Auswertung Reproduction_unshelled embryos_raw

Test design

Number of treatments (incl. control(s))	6
Duration of the test	28.0 d
Measurement variable	Reproduction - unshelled embryos
Test system	Potamopyrgus antipodarum

Reproduction - unshelled embryos of Potamopyrgus antipodarum as Dependent on Concentration and Time

Tab. 1: Reproduction - unshelled embryos [embryo number] of Potamopyrgus antipodarum as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (from Tabs Reproduction - unshelled embryos)

Treatm. [µg/L]	Control	1.060	3.540	10.500	36.300	105.000
28.0 d	7.50	8.00	7.80	8.70	8.70	5.70
	6.50	8.30	5.30	8.00	7.00	6.80
	7.20	6.20	5.00	7.30	8.30	7.50
	4.30	8.00	7.70	7.50	8.50	7.50
	6.20	6.80	8.00	8.30	8.70	7.00
	7.70	9.50	4.50	8.00	7.70	8.20
Mean:	6.57	7.80	6.38	7.97	8.15	7.12
Std.Dev.:	1.252	1.164	1.612	0.513	0.675	0.847
n:	6	6	6	6	6	6
CV:	19.061	14.929	25.249	6.433	8.277	11.904

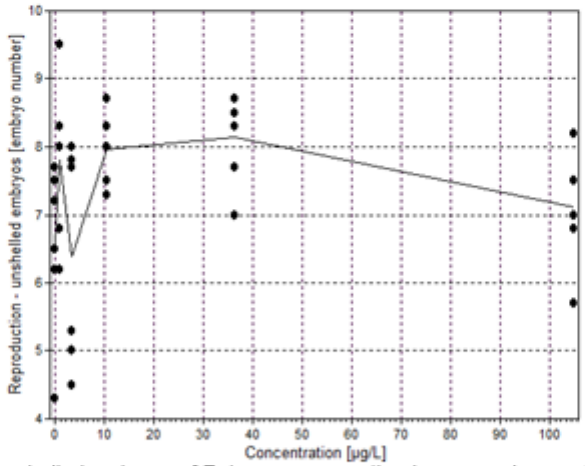


Fig. 1: Reproduction - unshelled embryos of Potamopyrgus antipodarum as observed under presence of the test item after 28.0 d.

Effective Concentrations (ECx) for Reproduction - unshelled embryos at 28.0 d

Reproduction - unshelled embryos [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Increase of reproduction - unshelled embryos caused by the test item after 28.0 d.

Treatm.[µg/L]	Mean	Std. Dev.	n	%Increase
Control	6.57	1.252	6	
1.060	7.80	1.164	6	18.782
3.540	6.38	1.612	6	-2.792
10.500	7.97	0.513	6	21.320
36.300	8.15	0.675	6	24.112
105.000	7.12	0.847	6	8.376

Probit analysis using linear max. likelihood regression

Tab. 3: Probit analysis using linear max. likelihood regression with reproduction - unshelled embryos at 28.0 d: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [µg/L]	Log(x)	%Decrease	n	Emp. Probit	Weight	Reg. Probit
1.060	0.025	0.00	1	-1.800	0.280	-1.128
1.060	0.025	0.00	1	-1.915	0.280	-1.128
1.060	0.025	5.58	1	-1.113	0.280	-1.128
1.060	0.025	0.00	1	-1.800	0.280	-1.128
1.060	0.025	0.00	1	-1.342	0.280	-1.128
1.060	0.025	0.00	1	-2.373	0.280	-1.128
3.540	0.549	0.00	1	-1.724	0.265	-1.153
3.540	0.549	19.29	1	-0.770	0.265	-1.153
3.540	0.549	23.86	1	-0.655	0.265	-1.153
3.540	0.549	0.00	1	-1.686	0.265	-1.153
3.540	0.549	0.00	1	-1.800	0.265	-1.153
3.540	0.549	31.47	1	-0.464	0.265	-1.153
10.500	1.021	0.00	1	-2.068	0.251	-1.175
10.500	1.021	0.00	1	-1.800	0.251	-1.175
10.500	1.021	0.00	1	-1.533	0.251	-1.175
10.500	1.021	0.00	1	-1.610	0.251	-1.175
10.500	1.021	0.00	1	-1.915	0.251	-1.175
10.500	1.021	0.00	1	-1.800	0.251	-1.175
36.300	1.560	0.00	1	-2.068	0.236	-1.201
36.300	1.560	0.00	1	-1.419	0.236	-1.201
36.300	1.560	0.00	1	-1.915	0.236	-1.201
36.300	1.560	0.00	1	-1.991	0.236	-1.201
36.300	1.560	0.00	1	-2.068	0.236	-1.201

36.300	1.560	0.00	1	-1.686	0.236	-1.201
105.000	2.021	13.20	1	-0.922	0.224	-1.223
105.000	2.021	0.00	1	-1.342	0.224	-1.223
105.000	2.021	0.00	1	-1.610	0.224	-1.223
105.000	2.021	0.00	1	-1.610	0.224	-1.223
105.000	2.021	0.00	1	-1.419	0.224	-1.223
105.000	2.021	0.00	1	-1.877	0.224	-1.223

excluded: value not in line with the chosen function

Inhibition greater than 100 or lower than 0% were replaced by 100.0% and 0%, respectively.

Parameters of the probit analysis

Tab. 4: Parameters of the probit analysis with reproduction - unshelled embryos at 28.0 d: Results of the regression analysis

Parameter	Value
Computation runs:	2
Slope b:	-0.11896
Intercept a:	-1.49909
Variance of b:	0.26559
Goodness of Fit	
Chi²:	1.12663
Degrees of freedom:	28
p(Chi²):	1.000
Log EC50:	-12.60170
SE Log EC50:	58.91579
g-Criterion:	3.16866
Residual Variance (Chi²/df):	0.04024
r²:	0.045
F:	1.324
p(F) (df: 1;28):	0.260

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0.100 data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No statistically significant concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significantly different from zero).
Due to the lacking concentration/response the shown ECx could not be valid.
Nonetheless, the user decided to show the ECx, confidence limits and graphs if possible.

Results of the probit analysis

Tab. 5: Results of the probit analysis with reproduction - unshelled embryos at 28.0 d: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	EC10	EC20	EC50
Value [µg/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 0.000 Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

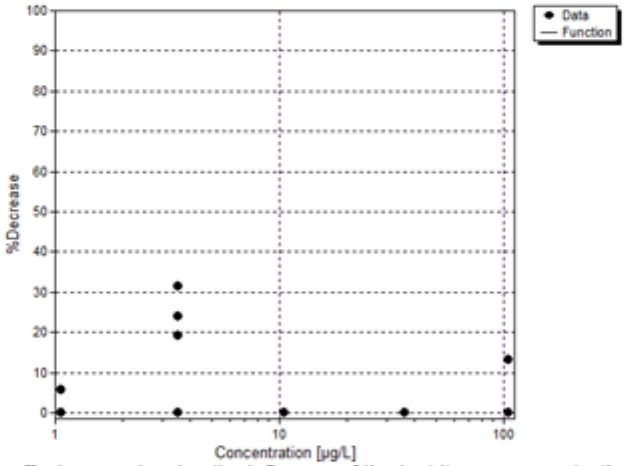


Fig. 2: Concentration-effect curve showing the influence of the test item on reproduction - unshelled embryos of the introduced Potamopyrgus antipodarum as observed after 28.0 d

Threshold concentrations (NOEC) for Reproduction - unshelled embryos at 28.0 d

Reproduction - unshelled embryos [embryo number] in Potamopyrgus antipodarum after 28.0 d.

Tab. 2: %Decrease of reproduction - unshelled embryos caused by the test item after 28.0 d.

Treatm.[ng/L]	Mean	Std. Dev.	n	%Decrease
Control	3.65	1.263	6	
6.970	4.32	1.107	6	-18.265
11.170	4.85	1.188	6	-32.877
25.600	3.97	0.472	6	-8.676
106.000	4.52	0.906	6	-23.744
332.000	4.10	0.787	6	-12.329

Statistical Characteristics of the Samples

Tab. 3: Statistical characteristics with reproduction - unshelled embryos at 28.0 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [ng/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	3.65	3.60	2.30	5.70	6	1.263	34.6	0.516	14.1	2.32	4.98
6.970	4.32	4.10	3.00	6.00	6	1.107	25.6	0.452	10.5	3.15	5.48
11.170	4.85	4.55	3.30	6.30	6	1.188	24.5	0.485	10.0	3.60	6.10
25.600	3.97	4.10	3.30	4.50	6	0.472	11.9	0.193	4.9	3.47	4.46
106.000	4.52	4.40	3.20	6.00	6	0.906	20.1	0.370	8.2	3.57	5.47
332.000	4.10	4.00	3.30	5.00	6	0.787	19.2	0.321	7.8	3.27	4.93

Shapiro-Wilk´s Test on Normal Distribution

Tab. 4: Shapiro-Wilk´s Test on Normal Distribution with reproduction - unshelled embryos at 28.0 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [ng/L]	Mean	s	n
Control	3.65	1.263	6
6.970	4.32	1.107	6
11.170	4.85	1.188	6
25.600	3.97	0.472	6
106.000	4.52	0.906	6
332.000	4.10	0.787	6

Results:

Number of residuals = 32; Shapiro-Wilk´s W = 0.973; p(W) = 0.595; p(W) is greater than the selected significance level of 0.010; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 5: Levene´s Test on Variance Homogeneity (with Residuals) with reproduction - unshelled embryos at 28.0 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	1.6150	5	0.3230	1.146	0.358
Residuals	8.4561	30	0.2819		
Total	10.071	35			

The Levene test indicates variance homogeneity (p > 0.010).

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 6: Trend analysis by contrasts (monotonicity of concentration/response) with reproduction - unshelled embryos at 28.0 d: Psi: sum of means weighted by contrasts; s(psi): standard error of psi; df: degrees of freedom; t: t-statistic; p(t): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	s(psi)	df	t	p(t)
Linear	-1.9667	3.3859	30	-0.581	0.283
Quadratic	5.3500	3.7091	30	1.442	0.080

The linear trend is not significant (p > 0.05) The quadratic trend is not significant (p > 0.05)

The analysis of contrasts did not reveal a linear trend, thus the selected Williams test was replaced by Dunnett test.

Dunnett`s Multiple t-test Procedure

Tab. 7: Dunnett´s multiple t-test procedure with reproduction - unshelled embryos at 28.0 d: Comparison of treatments with "Control". Significance was Alpha = 0.050, one-sided smaller (multiple level); Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [ng/L]	Mean	s	df	%MDD	t	t*	Sign.
Control	3.65	0.9913					
6.970	4.32	0.9913	30	-36.692	1.16	-2.34	-
11.170	4.85	0.9913	30	-36.692	2.10	-2.34	-
25.600	3.97	0.9913	30	-36.692	0.55	-2.34	-
106.000	4.52	0.9913	30	-36.692	1.51	-2.34	-
332.000	4.10	0.9913	30	-36.692	0.79	-2.34	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 332.000 ng/L.

C Appendix

C.1 Analytical report for Dienogest – Details of the method and results

C.1.1 Scope

The purpose of this analytical part of the study was to determine the analyte Dienogest in aqueous test medium (reconstituted water, cp. chapter 3.1.2) of an effect study on aquatic snails.

The quantitative measurements were done by liquid chromatography coupled to a triple quadruple mass spectrometer; the MS was operated in the tandem mass spectrometry mode (LC-MS/MS).

The active substance Dienogest (CAS no. 65928-58-7) was also used as the analytical standard; isotopically labelled Dienogest-d_6 was used as internal standard (IS).

C.1.2 Chemicals, reagents and analytical equipment

- Dienogest, active substance, Batch: S125103, Purity (HPLC): 99.88%, Stability: 3 years at -20°C, Article/catalog no.: S1251 (Selleckchem.com), see CoA in chapter D.1.1.
- Internal Standard, Dienogest-d_6, Lot: 055, Enrichment: 99%, Expiry Date: not specified, Article/product: D75937 (Medical Isotopes Inc.), see data sheet in chapter D.1.3.
- IS solution, solution of the internal standard Dienogest-d_6 in methanol, conc.: 10 µg/L
- Methanol, 'Methanol for LC-MS', Article No. 1428 (Th. Geyer)
- Acetonitrile, 'Acetonitrile for LC-MS', Article No. 2697 (Th. Geyer)
- Ammonium acetate, 'Optima LC-MS', Article No. 11317490 (Fisher Scientific)
- Purified water, produced with purification system Purelab® Ultra (ELGA LabWater)
- Solvent mixture 1: Mixture of 40 mL methanol and 10 mL acetonitrile
- Analytical balance XPE 205 DR (Mettler Toledo)
- Piston operated pipette, 'research 5000', variable volume selection (Eppendorf)
- 12 mL screw cap vials, 15 mm thread, 66 x 19 mm, clear glass; equipped with 15 mm screw caps with Teflon coated sealing disks (WiCom)
- Common laboratory equipment (volumetric flasks, glass beakers, Pasteur pipettes etc.)
- Screw top vials, approx. 2.0 mL capacity, clear glass; screw caps with Teflon coated sealing disks (WiCom)
- Microman™ pipettes (positive displacement), M25, M50 and M250 (Fisher Scientific)

C.1.3 Sampling and sample processing

Sampling

The samplings were done by the staff of the department ecotoxicology using piston operated pipettes.

To prevent degradation of the analyte and to minimize wall effects the aqueous samples were stabilized by adding methanol. Therefore, aliquots of accurately 0.50 mL methanol were filled into each 12 mL screw cap vials prior to the start of the sampling procedure.

Subsequent 5.00 mL of the aqueous test media were taken out of the test vessels and were pipetted into the screw top vials as well; the volume mix water/methanol was therefore 10+1 (v/v).

After vigorously mixing by hand the samples were stored in a freezer at a temperature of ≤ -18°C until analysis.

Sample processing

For direct LC-MS/MS measurement the samples were thawed and allowed to equilibrate to room temperature. Aliquots of 1.10 mL of the aqueous samples (water/methanol mixtures) were then filled into 2 mL screw top vials; afterwards 25.0 µL of the IS solution IS-IM-2a (cp. Table 42) were added and the vials were mixed by hand.

Finally 50 µL of the processed sample were injected into the LC-MS/MS system, the measuring method is described in detail in chapter C.1.4.

The remaining water/methanol mixtures were stored in a freezer at a temperature of ≤ -18 °C to allow a second measurement.

C.1.4 LC-MS/MS measurement

The quantitative determination of Dienogest was carried out by liquid chromatography and tandem mass spectrometry detection (LC-MS/MS) using positive electrospray ionization (ESI+). The measurement conditions and instrument settings are summarized below.

LC-MS/MS system

HPLC system:	Waters Acquity UPLC H-Class System
Mass spectrometer:	Waters LC-MS/MS Xevo TQ-S (triple quadruple system)
Software:	Waters MassLynx Ver. 4.1
Quantitation software:	Waters QuanLynx Ver. 4.1

LC parameters

Column:	Acquity UPLC BEH C18; 1.7 µm, 50 mm x 2.1 mm
Column temperature:	40°C
Injection volume:	50 µL
Flow rate:	0.30 mL/min
Mobile phase A:	1000 mL acetonitrile + 2mL 1 M ammonium acetate solution
Mobile phase B:	950 mL purified water + 50 mL acetonitrile + 2 mL 1 M ammonium acetate solution

Gradient program:	time [min]	solvent A [%]	solvent B [%]
	0.00	10	90
	0.20	20	90
	4.00	50	50
	4.10	100	0
	6.60	100	0
	6.61	10	90
	8.00	10	90

MS method (measurement conditions and instrument setting)

Type:	MRM	Ion mode:	ESI+
Span:	0.2 Da	Solvent delay 1:	0.10 – 3.90 min
Solvent delay 2:	4.90 – 7.95 min	End time:	8.00 min
Collision gas:	Argon		

Usage of MRM transition	Retention time [min]	Precursor ion [m/z]	Product ion [m/z]	Cone voltage [V]	Collision energy [eV]	Dwell time [s]
Analyte, quantitation ion	4.25	312.28	160.98	30	25	0.100
Analyte, qualifier ion		312.28	135.02	30	28	0.100
IS, quantitation ion	4.25	318.34	139.03	30	30	0.100
IS, qualifier ion		318.34	167.03	30	27	0.100

MS parameters

Source settings		Analyzer settings	
Capillary:	0.80 kV	LM 1 resolution:	2.9
Source Offset:	50.0 V	HM 1 resolution:	15.0
Source temperature:	150°C	Ion energy 1:	0.7
Desolvation temperature:	600°C	MS Mode Entrance:	1
Cone gas flow:	150 L/h	MS Mode Exit:	1
Desolvation gas flow:	1000 L/h	LM 2 resolution:	2.7
Collision gas flow:	0.18 L/min	HM 2 resolution:	15.0
Nebulizer gas flow:	7.00 bar	Ion energy 2:	2.0
		Gain (multiplier)	1.00

C.1.5 Calibration, quantification and calculation of the analytical results

Stock and intermediate solutions of the analyte and the internal standard

The stock solutions of active substance (analyte) Dienogest and the internal standard (IS) Dienogest-d_6 were prepared by exact weighing the standard compounds directly into separate volumetric flasks and by subsequent filling up to the ring mark with methanol, see Table 40.

Table 40: Preparation of stock solutions of the analyte and the internal standard.

Stock solution	Compound	Date of preparation	Weighed amount	Purity	Volumetric flask, nominal volume	Resulting concentration
S-1c	Dienogest	Nov.29, 2018	20.83 mg	99.88%	20 mL	1.040 g/L
S-1b	Dienogest	Sept. 06, 2018	20.17 mg	99.88%	20 mL	1.007 g/L
S-1d	Dienogest	Nov.19, 2019	20.33 mg	99.88%	20 mL	1.015 g/L
IS-1a	Dienogest-d6	Sept. 07, 2018	1.01 mg *	99%	5 mL	0.200 g/L

* Total quantity delivered

For the preparation of the calibration solutions intermediate solutions (IM) were prepared by pipetting aliquots of the analyte stock S-1a or the IM solution IM-1a into additional volumetric flasks and filling up to the mark with methanol, the resulting concentrations and the dilution scheme are given in Table 41.

Table 41: Preparation of analyte intermediate solution (IM set 1).

Analyte intermediate (IM) solution	Date of preparation	Used solution	Used volume	Volumetric flask, nominal volume	Resulting concentration
IM-1c	Nov.29, 2018	S-1c	48.07 µL	25 mL	2.00 mg/L
IM-2c	Nov.29, 2018	IM-1c	50.00 µL	20 mL	5.00 µg/L

For spiking the test media samples during sample processing (cp. chapter C.1.3) and preparation of the calibration solutions an IM solution of the IS was prepared by dilution with methanol, the resulting concentration and the dilution scheme is given in Table 42.

Table 42: Preparation of internal standard spiking solution.

IS intermediate (IM) solution	Date of preparation	Used solution	Used volume	Volumetric flask, nominal volume	Resulting concentration
IS-IM-1a	Sept. 06, 2018	IS-1a	50.00 µL	10 mL	1.00 mg/L
IS-IM-2a	Sept. 06, 2018	IS-IM-1a	250.0 µL	25 mL	10.0 µg/L

For the preparation of the QC standards an additional set of analyte IM solutions was prepared in methanol based on the analyte stock S-1b; the solutions, the dilution scheme and the resulting concentrations are given in Table 43.

On November 19, 2019, new QC standards were set. For this purpose the procedure from September 07, 2018 was repeated; the dilution scheme and the resulting concentrations are given in Table 44.

Table 43: Preparation of analyte intermediate solution (IM set 2).

Analyte intermediate (IM) solution	Date of preparation	Used solution	Used volume	Volumetric flask, nominal volume	Resulting concentration
IM-1b	Sept. 07, 2018	S-1b	49.64 µL	25 mL	2.00 mg/L
IM-2b	Sept. 03, 2018	IM-1b	50.00 µL	20 mL	5.00 µg/L

Table 44: Preparation of analyte intermediate solution (IM set 3).

Analyte intermediate (IM) solution	Date of preparation	Used solution	Used volume	Volumetric flask, nominal volume	Resulting concentration
IM-1d	Nov. 19, 2019	S-1d	49.25 µL	25 mL	2.00 mg/L
IM-2d	Nov. 19, 2019	IM-1d	50.00 µL	20 mL	5.00 µg/L

All prepared stock and intermediate solutions were stored at a temperature of approx. 4°C in a refrigerator.

Preparation of the calibration standards and the matrix calibration samples

Due to the expected higher stability of the analyte in an organic solvent the 'calibration standards' (calibration solutions) were prepared and stored in pure methanol.

Nine calibration standards (C-1c, C-2c, …) were prepared on November 29, 2018 in the concentration range from 1.00 to 500 ng/L by diluting the intermediate solution IM-2c in volumetric flasks with methanol; see pipetting plan in Table 45 (microman pipettes were used for this dilution step).

The 'matrix calibration samples' (CS-1, CS-2, …) were prepared afterwards by mixing 100 µL of the calibration standards and 25 µL of the IS solution IS-IM-2a with 1000 µL aqueous test medium in 2 mL screw top vials; the volume mix water/methanol was therefore 10+1 (v/v), this was the same solvent composition as it existed at the end of sample processing, cp. chapter C.1.3. The calibration samples were measured as described in chapter C.1.4.

All prepared calibration standards were stored at approximately 4°C in a refrigerator.

Table 45: Preparation of the calibration standards and the calibration samples, analyte Dienogest.

No. of the calibration solution	Volume solution IM-2c	Volumetric flask, nominal volume	Analyte concentration, calibration solution	No. of the calibration sample	Analyte concentration, calibration sample *
C-1c	20.00 µL	10 mL	10.0 ng/L	CS-1	1.00 ng/L
C-2c	50.00 µL	10 mL	25.0 ng/L	CS-2	2.50 ng/L
C-3c	100.0 µL	10 mL	50.0 ng/L	CS-3	5.00 ng/L
C-4c	250.0 µL	10 mL	125 ng/L	CS-4	12.5 ng/L
C-5c	500.0 µL	10 mL	250 ng/L	CS-5	25.0 ng/L

No. of the calibration solution	Volume solution IM-2c	Volumetric flask, nominal volume	Analyte concentration, calibration solution	No. of the calibration sample	Analyte concentration, calibration sample *
C-6c	1000 µL	10 mL	500 ng/L	CS-6	50.0 ng/L
C-7c	2000 µL	10 mL	1000 ng/L	CS-7	100 ng/L
C-8c	5000 µL	10 mL	2500 ng/L	CS-8	250 ng/L
C-9c	10000 µL	10 mL	5000 ng/L	CS-9	500 ng/L

*) Remark: The concentrations of the final calibration samples were related to the analyte amounts in the methanol/water mixture. They are lower than the actual concentrations as the added volumes of the calibration standards (CS-1, CS-2, ...) and the IS solution are not considered. These concentrations correspond to the concentrations of test samples to be analyzed by this method. For analysis of (aqueous) test samples equal amounts of the solvent used in the calibration standards are added to the test samples. So the test samples are treated in the same manner as the calibration samples and contained water and solvent at same concentrations (volume mixture methanol/water = 1+10, cp. chapter C.1.3).

This procedure was repeated on every measuring day and new matrix calibration functions were recorded.

Matrix calibration of the LC-MS/MS system and creating the calibration function

The LC-MS/MS system was calibrated for the analysis of the aqueous test samples by preparation and measuring of the prepared calibration samples (cp. previous chapters). Afterwards the chromatographic raw data were processed (integrated) using the Waters Quan-Lynx software. Subsequent the calibration functions was set up by the 'internal standard method' plotting the peak area ratios (PAR = integrated peak area analyte / integrated peak area IS) against the used analyte concentrations. With the received calibration data a linear regression calculation was performed.

Quantification and calculation of the analytical results

The LC-MS/MS quantification data were generated by processing the chromatographic raw data of the measured samples and by subsequent calculation of the quantification results ($C_{LC\text{-}MS/MS}$) using the respective matrix calibration function.

As the aqueous test samples (water) and the calibration samples were pre-treated (diluted) in the same way and were analyzed by direct injection into the LC-MS/MS system, the concentrations of the analyte in the aqueous test samples (C_W) were quantified directly from the relevant calibration function ($C_{LC\text{-}MS/MS} = C_W$).

Quality control

Two quality control (QC) standards were used for the verification of the basic calibration. The QC standard solutions were prepared analogous to calibration standards, but were based on separate weights of the analytical standard, cp. Table 40 (solutions S-1b and S-1d).

The QC standard solutions QC-1b and QC-2b were prepared on September 07, 2018 in concentrations of 50.0 ng/L and 2500 ng/L by diluting the IM-2b solution in separate volumetric flasks with methanol; see pipetting plan in Table 46.

On the day of measurement, the QC samples QC-S-1b and QC-S-2b were prepared by mixing 100 µL of the QC standard solutions with 25.0 µL of IS solution IS-IM-2a and 1000 µL aqueous test medium in screw top vials (volume mixture water/methanol = 10+1 (v+v). The analyte concentration in the QC samples were thus 5.00 ng/L and 250 ng/L, the QC samples were measured in turn as described in chapter C.1.4.

The prepared QC standard solutions were as well stored in a refrigerator.

Table 46: Preparation of the quality control (QC) standards and QC samples.

No. of the QC standard	Volume solution IM-2b	Volumetric flask, nominal volume	Analyte concentration, QC solutions	No. of the QC sample	Analyte concentration, QC samples *)
QC-1b	100.0 µL	10 mL	50.0 ng/L	QC-S-1b	5.00 ng/L
QC-2b	5000 µL	10 mL	2500 ng/L	QC S-2b	250 ng/L

*) The comment to Table 45 also applies to the analyte concentrations of the QC samples

The measurement intervals of the QC standards were every twentieth sample, but at least once a measurement day.

As already mentioned, new QC standards were produced on November 19, 2019 (QC-1d and QC-2d). According to the described procedure, new QC samples were prepared and measured using the solution IM-2d; the concentrations were again 5.00 ng/L and 250 ng/L.

C.1.6 Results

Matrix calibration function

The basic calibration function (response type: internal standard) used for the quantification of Dienogest in the measured aqueous test medium samples is shown in Figure 6; the calibration function was calculated by linear regression analysis using the Waters QuanLynx software to:

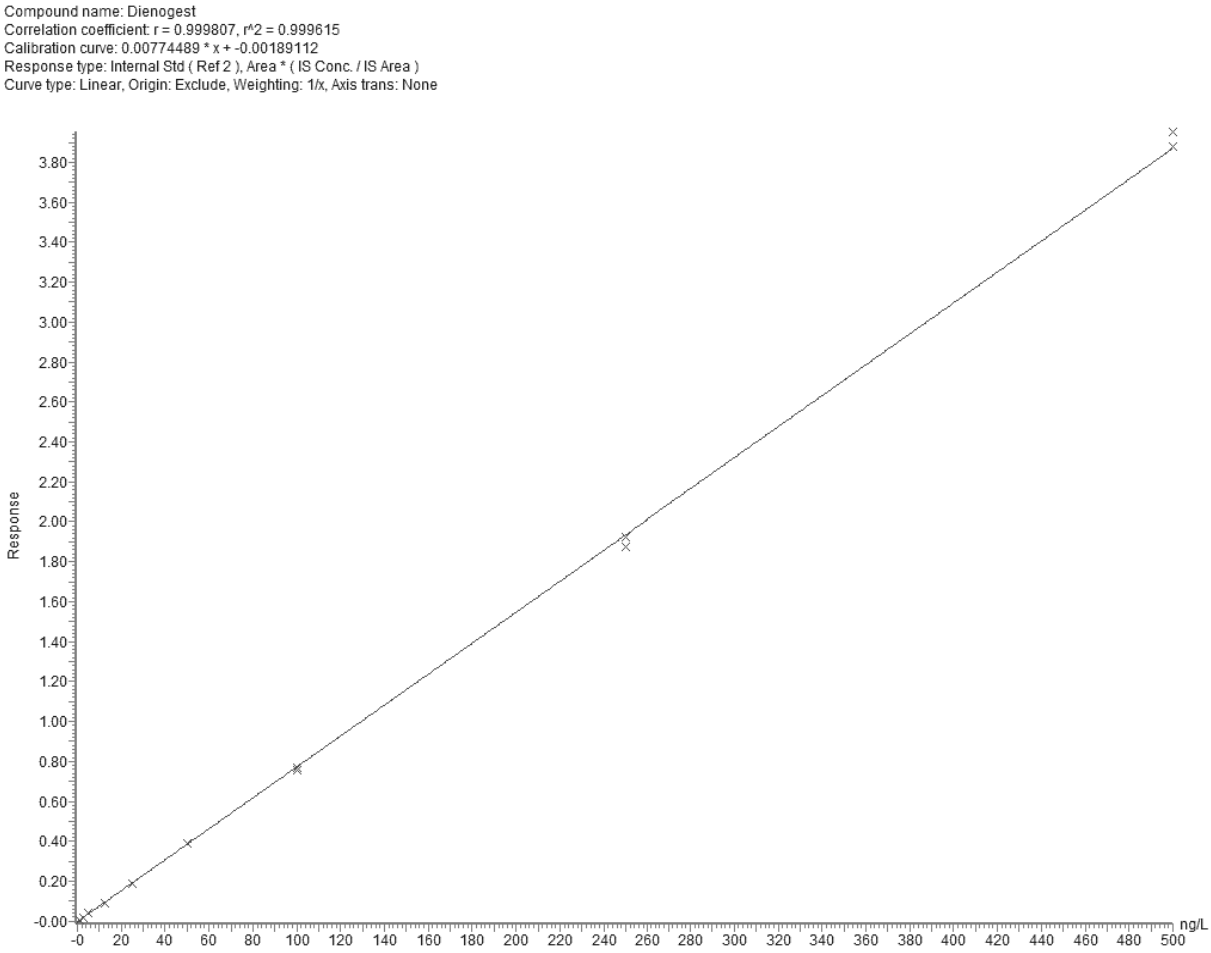
Function: $PAR = 0.007745 \bullet C_{Cal} - 0.001891$ $r^2 = 0.9996$

PAR = Peak area ratio
C_{Cal} = Analyte concentration of the calibration solutions
r^2 = Coefficient of determination

Linearity

Using the linear regression model, the coefficient of determination for Dienogest was calculated to 0.9996. As the calculated r^2-values were very close to 1, the linearity of the calibration functions was accepted.

Figure 6: Basic calibration function of Dienogest as measured on September 02, 2019 (twofold injection).



Results of the analyzed samples

The Dienogest concentrations in aqueous test media samples were assessed by chemical analysis, samples of all treatments and the controls were analyzed at test start and at the end of the test (fresh and aged test medium). In the meantime, only the controls, as well as the lowest and highest test concentrations were measured.

The analytical results are listed in Table 47; in addition to samples information, the quantification data, the measured analyte concentrations in water (C_W) and the calculated 'Percent of nominal' values were inserted into the table.

Table 47: Analyzed Dienogest concentrations (C_W) and corresponding 'percent of nominal' values (mass transition m/z 312.28 ⇨ m/z 161.13).

Sampling, date and time (2019)	Treatment	Nominal Dienogest conc.	Aging	LC-MS/MS quantific. data, CLC-MS/MS	Measured analyte conc., CW	Percent of nominal
July 10, day 0	Control	-	Fresh	-	-	-
	Conc. 1	3.2 ng/L		6.960	6.96	217.5
	Conc. 2	10 ng/L		10.753	10.8	107.5
	Conc. 3	32 ng/L		25.230	25.2	78.8
	Conc. 4	100 ng/L		108.125	108	108.1
	Conc. 5	320 ng/L		334.384	334	104.5
July 12, day 2	Control	-	Aged	-	-	-
	Conc. 1	3.2 ng/L		6.952	6.95	217.3
	Conc. 2	10 ng/L		11.171	11.2	111.7
	Conc. 3	32 ng/L		25.414	25.4	79.4
	Conc. 4	100 ng/L		106.920	107	106.9
	Conc. 5	320 ng/L		332.992	333	104.1
July 17, day 7	Control	-	Fresh	-	-	-
	Conc. 1	3.2 ng/L		6.747	6.75	210.8
	Conc. 5	320 ng/L		340.171	340	106.3
July 19, day 9	Control	-	Aged	-	-	-
	Conc. 1	3.2 ng/L		7.080	7.08	221.3
	Conc. 5	320 ng/L		344.148	344	107.5
July 24, day 14	Control	-	Fresh	-	-	-
	Conc. 1	3.2 ng/L		6.745	6.75	210.8
	Conc. 5	320 ng/L		331.936	332	103.7
July 26, day 16	Control	-	Aged	-	-	-
	Conc. 1	3.2 ng/L		6.591	6.59	206.0
	Conc. 5	320 ng/L		322.140	322	100.7
July 31, day 21	Control	-	Fresh	-	-	-
	Conc. 1	3.2 ng/L		6.961	6.96	217.5
	Conc. 5	320 ng/L		304.347	304	95.1
August 02, day 23	Control	-	Aged	0.000	-	-
	Conc. 1	3.2 ng/L		5.891	5.89	184.1

Sampling, date and time (2019)	Treatment	Nominal Dienogest conc.	Aging	LC-MS/MS quantific. data, CLC-MS/MS	Measured analyte conc., CW	Percent of nominal
	Conc. 5	320 ng/L		299.911	300	93.7
August 05, day 26	Control	-	Fresh	-	-	-
	Conc. 1	3.2 ng/L		7.306	7.31	228.3
	Conc. 2	10 ng/L		11.518	11.5	115.2
	Conc. 3	32 ng/L		28.740	28.7	89.8
	Conc. 4	100 ng/L		107.196	107	107.2
	Conc. 5	320 ng/L		334.801	335	104.6
August 07, day 28	Control	-	Aged	-	-	-
	Conc. 1	3.2 ng/L		6.663	6.66	208.2
	Conc. 2	10 ng/L		11.245	11.2	112.5
	Conc. 3	32 ng/L		23.403	23.4	73.1
	Conc. 4	100 ng/L		100.983	101	101.0
	Conc. 5	320 ng/L		327.469	327	102.3

Quality control

Each set of QC samples was measured once during the study. The QC samples of set1 (aged QC standards, QC-1b and QC-2b) were measured as part of sample analysis on September 10, 2019 and the QC samples of set 2 (new QC standards, QC-1d and QC-2d) were measured on November 20, 2019 to confirm the stability of the calibration standards.

The recoveries for set 1 (Dienogest conc. 5.00 ng/L) were 96.0% and 95.4% and for set 2 (conc. 250 ng/L) 100.0% and 99.1%. The quality control data shows that the measurements were done with high accuracy and precision and that the calibration standards (calibration solutions) can be stored stable over a period of approx. 1 year.

C.1.7 Representative LC-MS/MS chromatograms

Typical LC-MS/MS chromatograms of calibration samples, controls and test media samples are shown in Figure 7 to Figure 14.

Each figure shows four ion chromatograms in stacked windows (top-down):

Ion chromatogram	Mass transition
Dienogest, quantification ion	m/z 312.28 ⇨ m/z 160.98
Dienogest, qualifier ion	m/z 312.28 ⇨ m/z 135.02
Dienogest-d_6 (IS), quantification ion	m/z 318.34 ⇨ m/z 139.03
Dienogest-d_6 (IS), qualifier ion	m/z 318.34 ⇨ m/z 167.03

The dashed line in some chromatograms shows the baseline of the integrated peaks executed by automatic integration using the Waters QuanLynx software. However, the grey highlighted part of the chromatographic peak reflects the manually integrated peak area; this corrected peak area was used for quantification of the analyte.

The retention time (t_R) for Dienogest was approximately 4.25 min.

Figure 7: Calibration sample CS-1 measured on Sept. 02, 2019; Dienogest conc.: 1.00 ng/L.

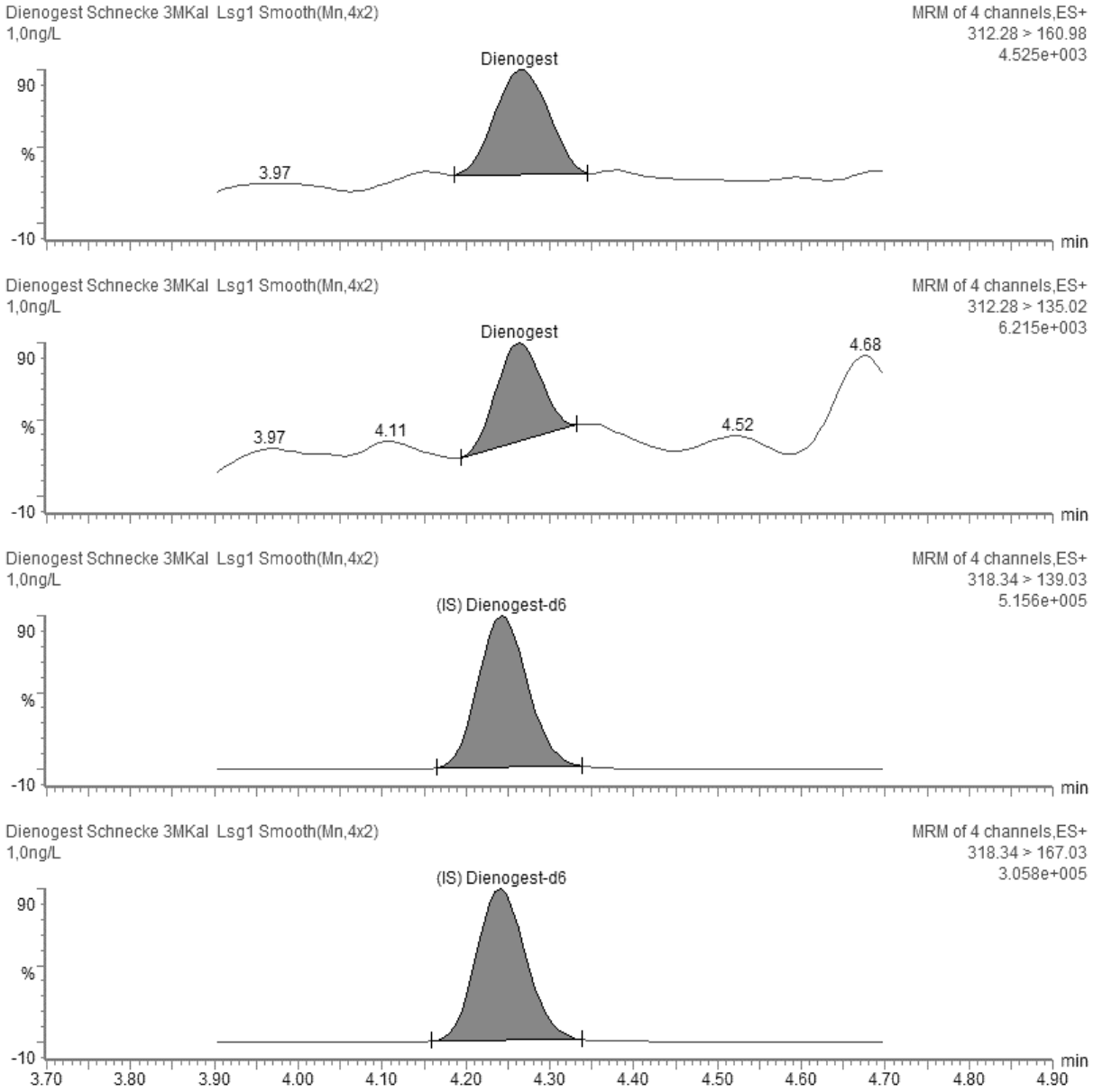


Figure 8: Calibration sample CS-9 measured on Sept. 02, 2019; Dienogest conc.: 500 ng/L.

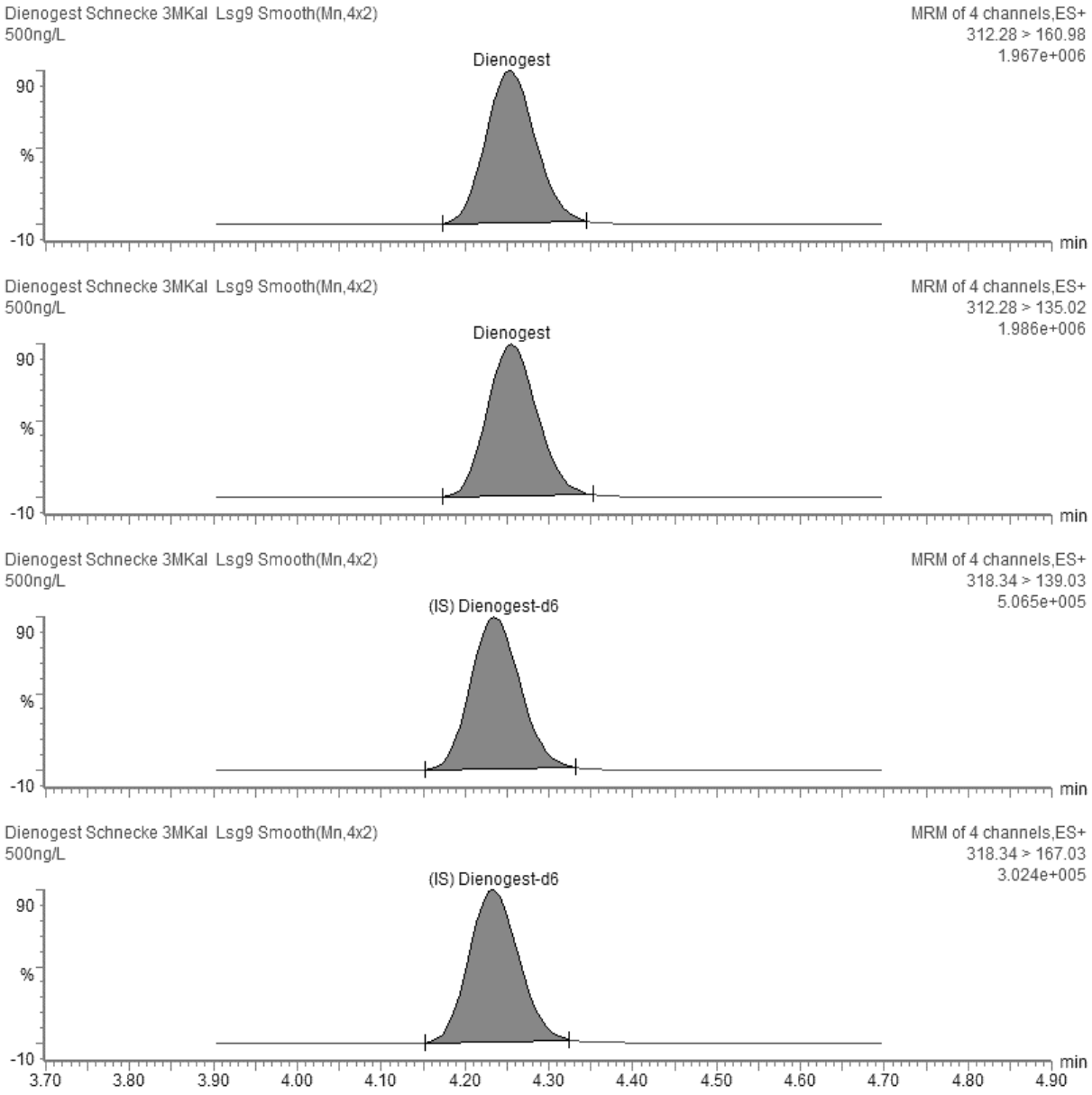


Figure 9: Control sample, fresh test medium, sampling time: test start (July 10, 2019).

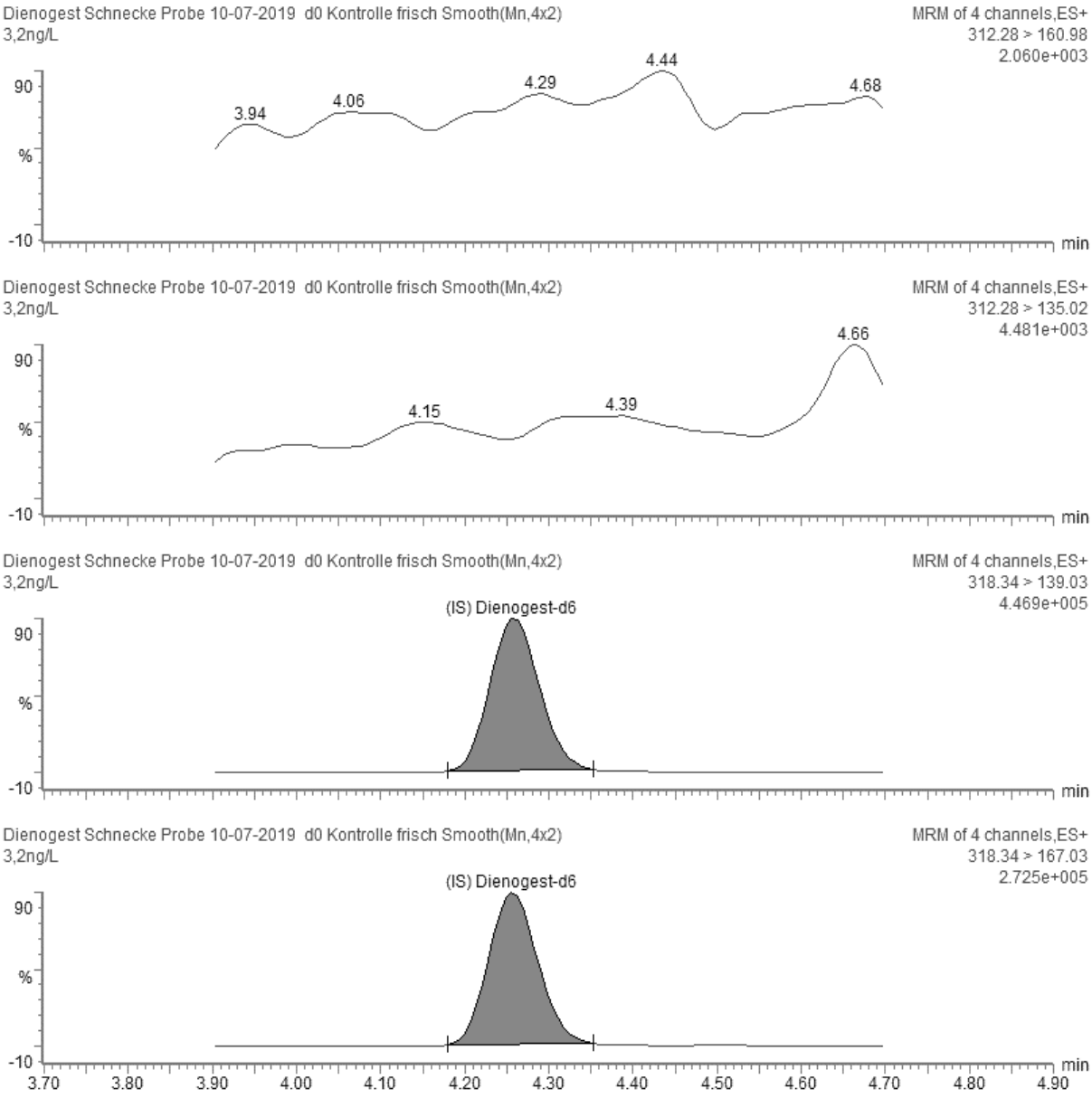


Figure 10: Control sample, aged test medium, sampling time: end of test (August 07, 2019).

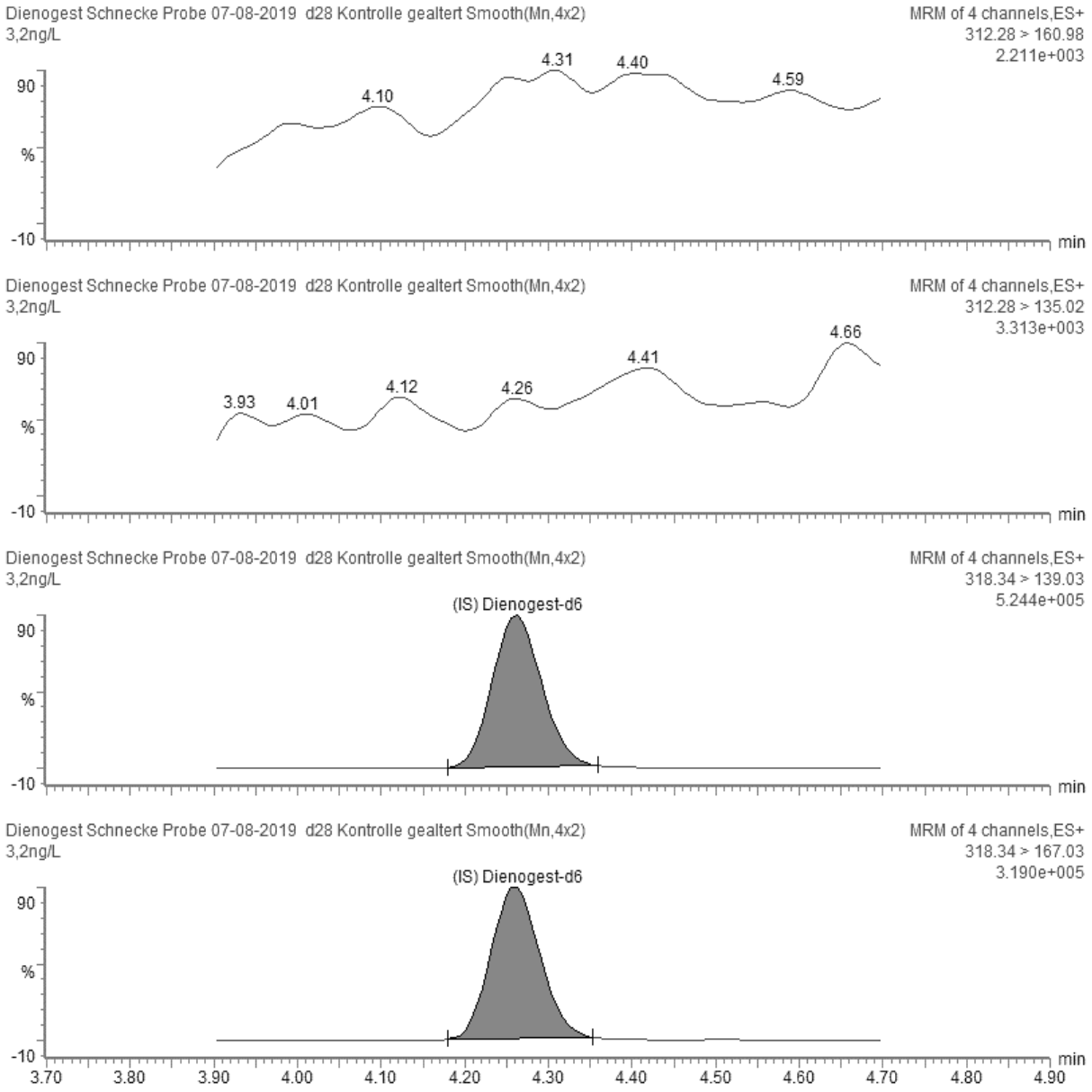


Figure 11: Test media sample, fresh test medium, treatment 1, nominal Dienogest conc.: 3.2 ng/L, sampling time: test start (July 10, 2019).

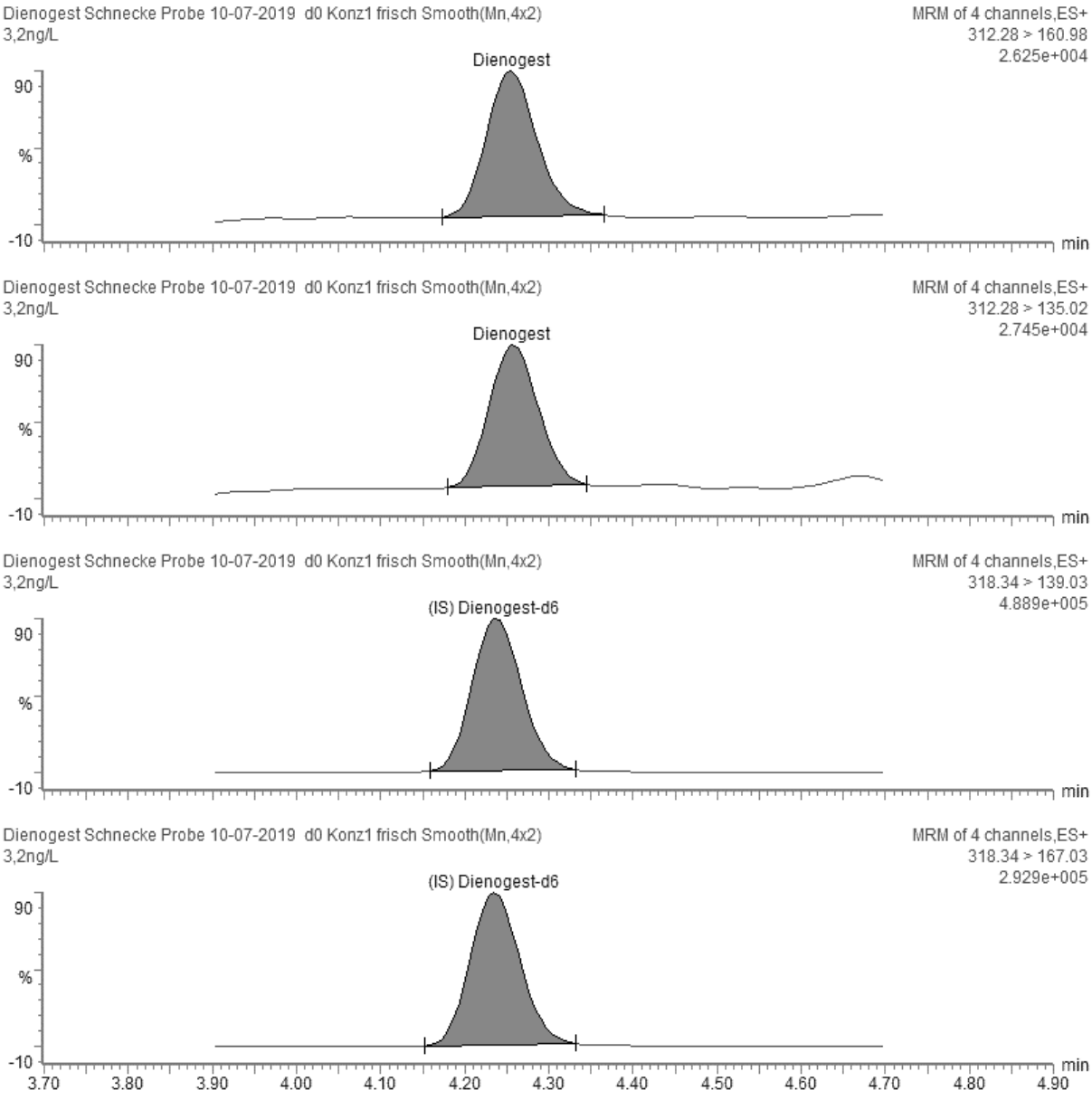


Figure 12: Test media sample, aged test medium, treatment 1, nominal Dienogest conc.: 3.2 ng/L, sampling time: end of test (August 07, 2019).

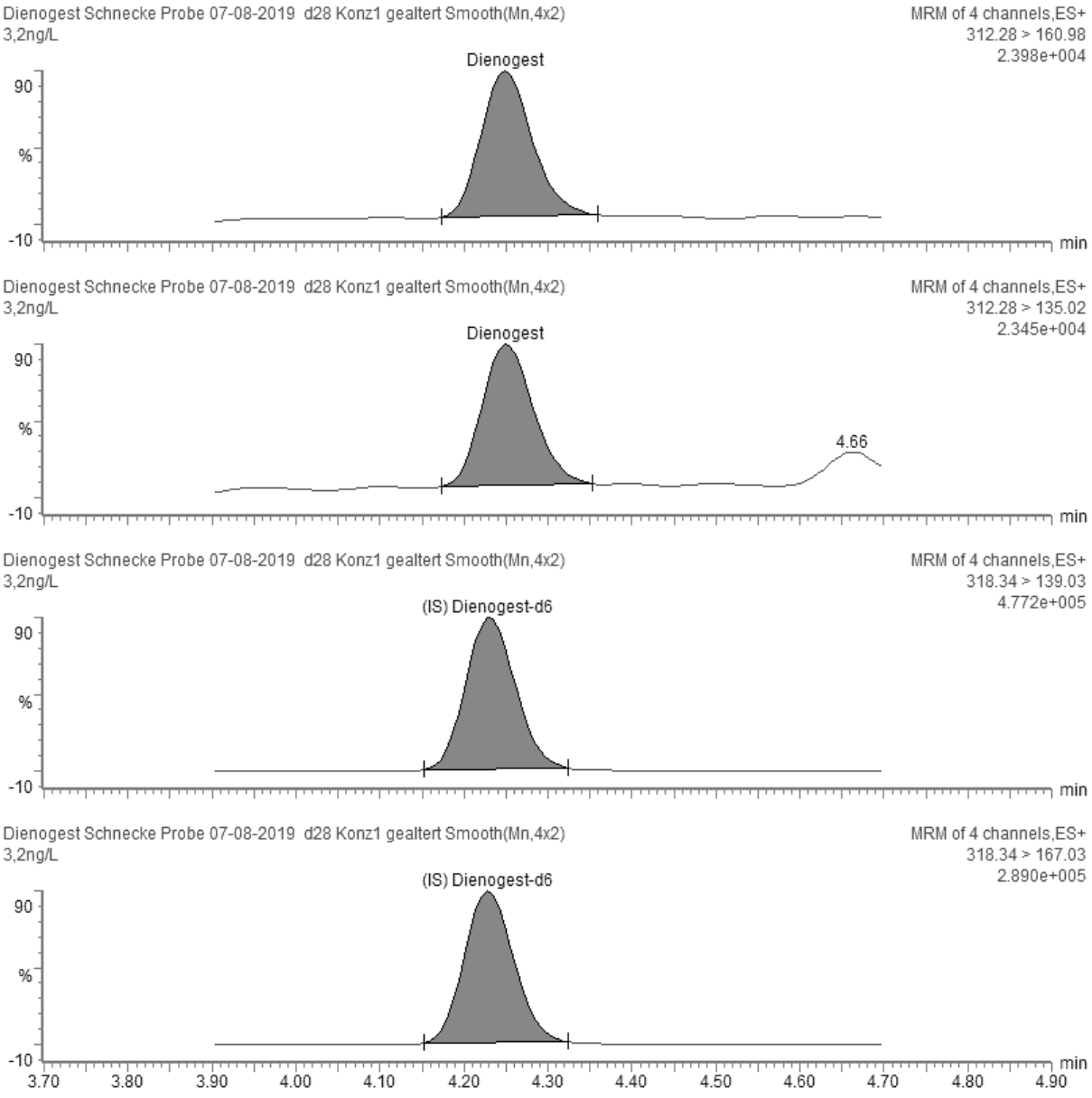


Figure 13: Test media sample, fresh test medium, treatment 5, nominal Dienogest conc.: 320 ng/L, sampling time: test start (July 10, 2019).

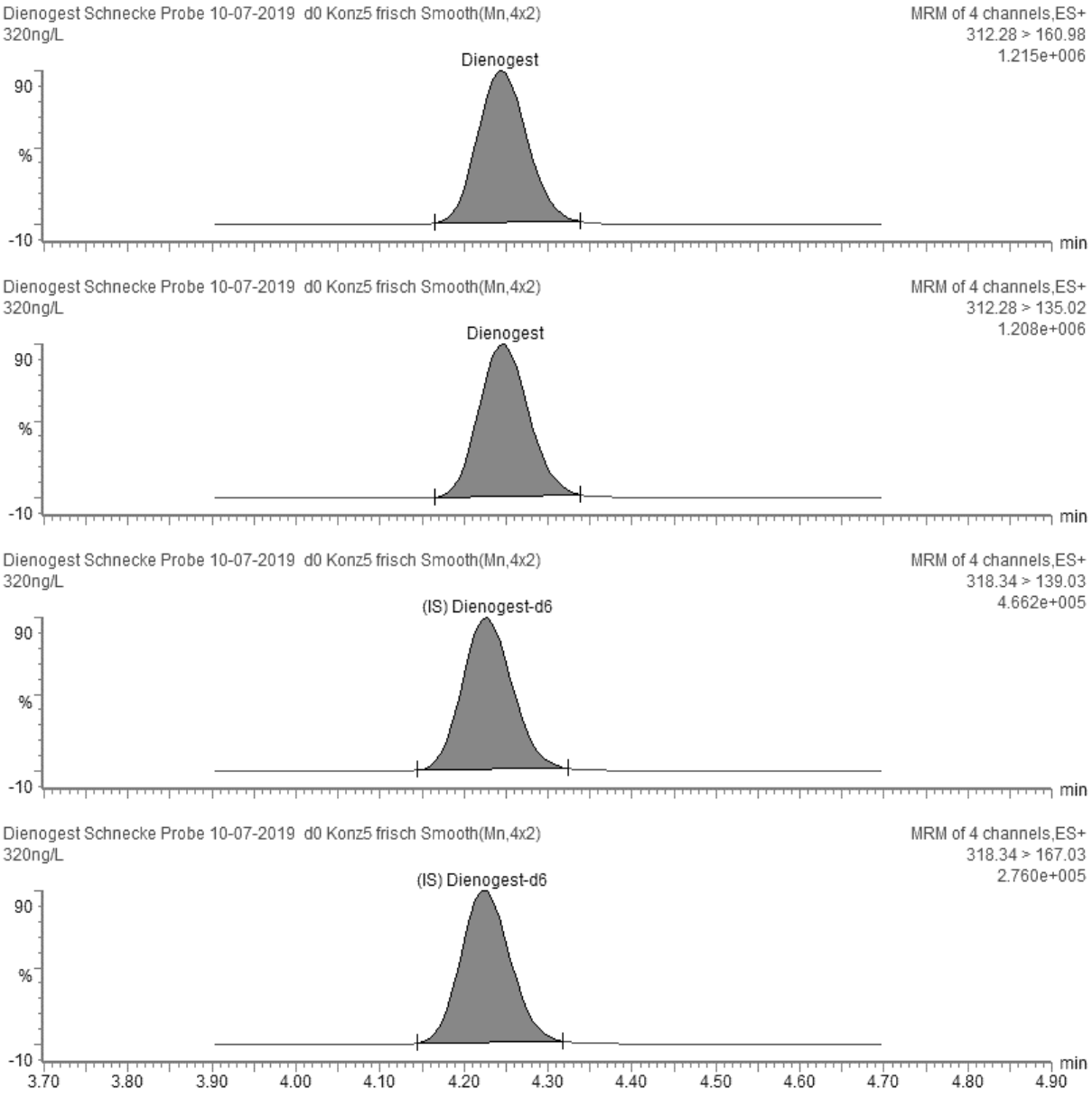
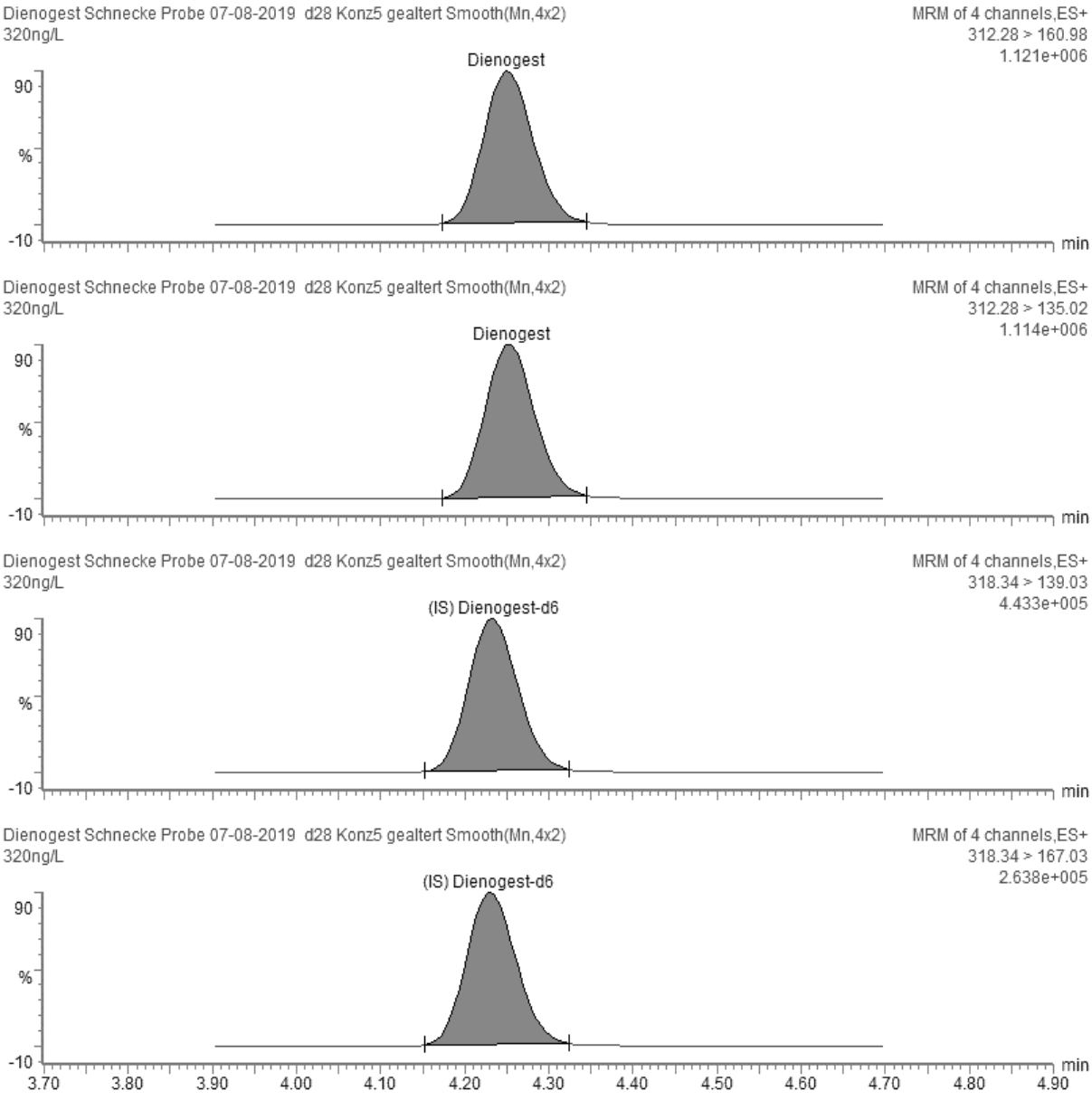


Figure 14: Test media sample, aged test medium, treatment 5, nominal Dienogest conc.: 320 ng/L, sampling time: end of test (August 07, 2019).



C.2 Analytical report for Dexamethasone – Details of the method and results

C.2.1 Scope

The purpose of this analytical part of the study was to determine the analyte Dexamethasone in aqueous test medium (reconstituted water, cp. chapter 3.1.2) of an effect study on aquatic snails.

The quantitative measurements were done by liquid chromatography coupled to a triple quadruple mass spectrometer; the MS was operated in the tandem mass spectrometry mode (LC-MS/MS).

The active substance Dexamethasone (CAS no. 50-02-2) was also used as the analytical standard; isotopically labelled Dexamethasone-d_4 (Dexamethasone-4,6α,21,21-d_4) was used as internal standard (IS).

C.2.2 Chemicals, reagents and analytical equipment

- Analytical standard Dexamethasone, Batch: BCBW7684, Purity (HPLC): 100 area%, Recommended retest date: FEB 2013, Product no.: D1756 (Sigma-Aldrich), see CoA in chapter D.1.4.
- Internal Standard, Dexamethasone-d_4, Lot no.: X-473, Chemical purity: 98.2%, Expiration: re-analyzed after three years, Product no.: D-5559 (CDN Isotopes), see data sheet in chapter D.1.5.
- IS solution, solution of the internal standard Dexamethasone-d_4 in methanol, conc.: 200 µg/L
- Methanol, 'Methanol for LC-MS', Article No. 1428 (Th. Geyer)
- Ammonium acetate, 'Optima LC-MS', Article No. 11317490 (Fisher Scientific)
- Formic acid, 'Optima LC-MS', Article No. 10596814 (Fisher Scientific)
- Purified water, produced with purification system Purelab® Ultra (ELGA LabWater)
- Solvent mixture 1: Mixture of 100 mL purified water and 20 mL methanol
- Analytical balance XPE 205 DR (Mettler Toledo)
- Piston operated pipette, 'research 5000', variable volume selection (Eppendorf)
- 12 mL screw cap vials, 15 mm thread, 66 x 19 mm, clear glass; equipped with15 mm screw caps with Teflon coated sealing disks (WiCom)
- Common laboratory equipment (volumetric flasks, glass beakers, Pasteur pipettes etc.)
- Screw top vials, approx. 2.0 mL capacity, clear glass; screw caps with Teflon coated sealing disks (WiCom)
- Microman™ pipettes (positive displacement), M25, M50 and M250 (Fisher Scientific)

C.2.3 Sampling and sample processing

Sampling

The samplings were done by the staff of the department ecotoxicology using piston operated pipettes.

To prevent degradation of the analyte and to minimize wall effects the aqueous samples were stabilized by adding methanol. Therefore, aliquots of accurately 1.00 mL methanol were filled into each 12 mL screw cap vials prior to start of the sampling procedure.

Subsequent 5.00 mL of the aqueous test media were taken out of the test vessels and were pipetted into the screw top vials as well; the volume mix water/methanol was therefore 5+1 (v/v).

After vigorously mixing by hand the samples were handed over to the chemical laboratory for immediate analysis; if this was not possible the samples were stored in a freezer at a temperature of ≤ -18°C until analysis.

Sample processing

For direct LC-MS/MS measuring the samples were thawed and allowed to equilibrate to room temperature. Aliquots of 1.20 mL of the aqueous samples (water/methanol mixtures) were then filled into 2 mL screw top vials; afterwards 50.0 µL of the IS solution IS-IM-1a (cp. Table 50) were added and the vials were mixed by hand.

If necessary, aliquots of the aqueous samples (water/methanol mixtures) were diluted with solvent mixture 1 to generate analyte concentrations which corresponds to concentration range of the basic calibration (dilution factor (D_F) of undiluted samples = 1).

Finally 100 µL of the processed sample were injected into the LC-MS/MS system, the measuring method is described in detail in chapter C.2.4.

The remaining water/methanol mixtures were stored in a freezer at a temperature of ≤ -18 °C to allow a second measurement.

C.2.4 LC-MS/MS measurement

The quantitative determination of Dexamethasone was carried out by liquid chromatography and tandem mass spectrometry detection (LC-MS/MS) using positive electrospray ionization (ESI+). The measurement conditions and instrument settings are summarized below.

LC-MS/MS system

HPLC system:	Waters 2695
Mass spectrometer:	Waters LC-MS/MS Quattro Micro (triple quadruple system)
Software:	Waters MassLynx Ver. 4.1
Quantitation software:	Waters QuanLynx Ver. 4.1

LC parameters

Column:	Phenomenex Gemini C18; 5 µm, 150 mm x 3 mm
Guard Column:	Phenomenex Gemini C18; 5 µm, 4 mm x 3 mm
Column temperature:	30°C
Injection volume:	100 µL

Flow rate:	0.50 mL/min
Mobile phase A:	1000 mL methanol + 2mL 1 M ammonium acetate solution
Mobile phase B:	900 mL purified water + 100 mL methanol + 2 mL 1 M ammonium acetate solution
Mobile phase C:	1000 mL methanol + 2mL 1 M formic acid

Gradient program:	time [min]	solvent A [%]	solvent B [%]	solvent C [%]
	0.00	45	50	5.0
	1.00	45	50	5.0
	3.00	95	0	5.0
	5.00	95	0	5.0
	5.20	45	50	5.0
	8.00	45	50	5.0

MS method (measurement conditions and instrument setting)

Type:	MRM	Ion mode:	ESI+
Span:	0.2 Da	Solvent delay 1:	0.10 – 4.50 min
Solvent delay 2:	7.00 – 8.00 min	End time:	8.00 min
Collision gas:	Argon		

Usage of MRM transition	Retention time [min]	Precursor ion [m/z]	Product ion [m/z]	Cone voltage [V]	Collision energy [eV]	Dwell time [s]
Analyte, quantitation ion	5.3	393.23	373.17	15	10	0.100
Analyte, qualifier ion		393.23	355.17	15	12	0.100
IS, quantitation ion	5.3	397.24	377.17	15	10	0.100
IS, qualifier ion		397.24	359.17	15	12	0.100

MS parameters

Source settings		Analyzer settings	
Capillary:	1.00 kV	LM 1 resolution:	13.7
Cone:	15.00 V	HM 1 resolution:	13.7
Extractor:	2.00 V	Ion energy 1:	0.5
RF Lens:	0.1 V	MS Mode Entrance:	1
Source Temperature [°C]:	120°C	MS Mode Exit:	1
Desolvation Temperature:	350°C	LM 2 resolution:	13.7
Cone Gas Flow:	100 L/h	HM 2 resolution:	13.7
Desolvation Gas Flow:	600 L/h	Ion energy 2:	1.0
		Multiplier (V):	650

C.2.5 Calibration, quantification and calculation of the analytical results

Stock and intermediate solutions of the analyte and the internal standard

The stock solutions of analytical standard Dexamethasone and the internal standard (IS) Dexamethasone-d_4 were prepared by exact weighing the standard compounds directly into separate volumetric flasks and by subsequent filling up to the ring mark with methanol, see Table 48.

Table 48: Preparation of stock solutions of the analyte and the internal standard.

Stock solution	Compound	Date of preparation	Weighed amount	Purity	Volumetric flask, nominal volume	Resulting concentration
S-1a	Dexamethasone	Aug. 29, 2019	20.40 mg	100%	20 mL	1.020 g/L
S-1b	Dexamethasone	Sept. 19, 2019	10.60 mg	100%	20 mL	0.530 g/L
IS-1a	Dexamethasone-d4	Sept. 18, 2019	5 mg *	98.2%	5 mL	0.986 g/L

* Total quantity delivered

For the preparation of the calibration solutions intermediate solutions (IM) were prepared by pipetting aliquots of the analyte stock S-1a or the IM solution IM-1a into additional volumetric flasks and filling up to the mark with methanol, the resulting concentrations and the dilution scheme are given in Table 49.

Table 49: Preparation of analyte intermediate solution (IM set 1).

Analyte intermediate (IM) solution	Date of preparation	Used solution	Used volume	Volumetric flask, nominal volume	Resulting concentration
IM-1a	Sept 19, 2019	S-1a	49.02 µL	10 mL	5.00 mg/L
IM-2a	Sept 19, 2019	IM-1a	400.0 µL	10 mL	200.0 µg/L

For spiking the test media samples during sample processing (cp. chapter C.2.3) and preparation of the calibration solutions an IM solution of the IS was prepared by dilution with methanol, the resulting concentration and the dilution scheme is given in Table 50.

Table 50: Preparation of internal standard spiking solution.

IS intermediate (IM) solution	Date of preparation	Used solution	Used volume	Volumetric flask, nominal volume	Resulting concentration
IS-IM-1a	Sept 19, 2019	IS-1a	10.14 µL	50 mL	0.200 mg/L

For the preparation of the QC standards an additional set of analyte IM solutions was prepared in methanol based on the analyte stock S-2a; the solutions, the dilution scheme and the resulting concentrations are given in Table 51.

Table 51: Preparation of analyte intermediate solution (IM set 2).

Analyte intermediate (IM) solution	Date of preparation	Used solution	Used volume	Volumetric flask, nominal volume	Resulting concentration
IM-3a	Sept 19, 2019	S-2a	37.74 µL	25 mL	2.00 mg/L
IM-4a	Sept 19, 2019	IM-3a	250.0 µL	20 mL	50.0 µg/L

All prepared stock and intermediate solutions were stored at a temperature of approx. 4°C in a refrigerator.

Preparation of the calibration standards and the matrix calibration samples

Due to the expected higher stability of the analyte in an organic solvent the 'calibration standards' (calibration solutions) were prepared and stored in pure methanol.

Eighth calibration standards (C-1a, C-2a, …) were prepared on September 19, 2019 in the concentration range from 0.400 to 250 µg/L by diluting the intermediate solutions IM-1a or IM-2a in volumetric flasks with methanol; see pipetting plan in Table 52 (microman pipettes were used for this dilution step).

The 'basic calibration samples' (CS-1, CS-2, …) were prepared afterwards by mixing 200 µL of the calibration standards and 50 µL of the IS solution IS-IM-1a with 1000 µL purified water in 2 mL screw top vials; the volume mix water/methanol was therefore = 5+1 (v/v), this was the same solvent composition as it existed at the end of sample processing, cp. chapter C.2.3. The calibration samples were measured as described in chapter C.2.4.

All prepared calibration standards were stored at approximately 4°C in a refrigerator.

Table 52: Preparation of the calibration standards and the calibration samples, analyte Dexamethason

No. of the calibration solution	Volume solution IM-1a	Volume solution IM-2a	Volumetric flask, nominal volume	Analyte concentration, calibration solution	No. of the calibration sample	Analyte concentration, calibration sample *
C-1a	-	20.00 µL	10 mL	0.400 µg/L	CS-1	0.080 µg/L
C-2a	-	125.0 µL	10 mL	2.50 µg/L	CS-2	0.500 µg/L
C-3a	-	250.0 µL	10 mL	5.00 µg/L	CS-3	1.00 µg/L
C-4a	-	500.0 µL	10 mL	10.0 µg/L	CS-4	2.00 µg/L
C-5a	50.00 µL	-	10 mL	25.0 µg/L	CS-5	5.00 µg/L
C-6a	100.0 µL	-	10 mL	50.0 µg/L	CS-6	10.0 µg/L
C-7a	250.0 µL	-	10 mL	125 µg/L	CS-7	25.0 µg/L
C-8a	500.0 µL	-	10 mL	250 µg/L	CS-8	50.0 µg/L

*) Remark: The concentrations of the final calibration samples were related to the analyte amounts in the methanol/water mixture. They are lower than the actual concentrations as the added volumes of the calibration standards (CS-1, CS-2, …and the IS solution are not considered. These concentrations correspond to the concentrations of test samples to be analyzed by this method. For analysis of (aqueous) test samples equal amounts of the solvent used in the calibration standards are added to the test samples. So the test samples are treated in the same manner as the calibration samples and contained water and solvent at same concentrations (volume mixture methanol/water = 1+5, cp. chapter C.2.3).

This procedure was repeated on every measuring day and new matrix calibration functions were recorded.

Basic calibration of the LC-MS/MS system and creating the calibration function

The LC-MS/MS system was calibrated for the analysis of the aqueous test samples by preparation and measuring of the prepared calibration samples (cp. previous chapters). Afterwards the chromatographic raw data were processed (integrated) using the Waters Quan-Lynx software. Subsequent the calibration functions was set up by the 'internal standard method' plotting the peak area ratios (PAR = integrated peak area analyte / integrated peak area IS) against the used analyte concentrations. With the received calibration data a linear regression calculation was performed.

Quantification and calculation of the analytical results

The LC-MS/MS quantification data were generated by processing the chromatographic raw data of the measured samples and by subsequent calculation of the quantification results ($C_{LC\text{-}MS/MS}$) using the respective matrix calibration function.

As the aqueous test samples (water) and the calibration samples were pre-treated (diluted) in the same way and were analyzed by direct injection into the LC-MS/MS system, the concentrations of the analyte in the aqueous test samples (C_W) were quantified directly from the relevant calibration function ($C_{LC\text{-}MS/MS} = C_W$).

Quality control

Two quality control (QC) standards were used for the verification of the basic calibration. The QC standard solutions were prepared analogous to calibration standards, but were based on separate weights of the analytical standard, cp. Table 48 (solutions S-1b).

The QC standard solutions QC-1a and QC-2a were prepared on September 19, 2019 in concentrations of 10.0 µg/L and 200 µg/L by diluting the IM-3a solution (2.00 mg Dexamethasone/L) in separate volumetric flasks with methanol; see pipetting plan in Table 53.

On the day of measurement, the QC samples QC-S-1a and QC-S-2a were prepared by mixing 200 µL of the QC standard solutions with 50.0 µL of IS solution IS-IM-1a and 1000 µL purified water in screw top vials (volume mixture water/methanol = 5+1 (v+v). The analyte concentration in the QC samples were thus 2.00 µg/L and 40.0 µg/L, the QC samples were measured in turn as described in chapter C.2.4.

The prepared QC standard solutions were as well stored in a refrigerator.

Table 53: Preparation of the quality control (QC) standards and QC samples.

No. of the QC standard	Volume solution IM-3a	Volumetric flask, nominal volume	Analyte concentration, QC solutions	No. of the QC sample	Analyte concentration, QC samples *)
QC-1a	50.0 µL	10 mL	10.0 µg/L	QC-S-1a	2.00 µg/L
QC-2a	1000 µL	10 mL	200 µg/L	QC S-2a	40.0 µg/L

*) The comment to Table 52 also applies to the analyte concentrations of the QC samples

The measurement intervals of the QC standards were every twentieth sample, but at least once a measurement day.

C.2.6 Results

Matrix calibration function

The basic calibration function (response type: internal standard) used for the quantification of Dexamethasone in the measured aqueous test medium samples was measured on October 02, 2019 and is shown in Figure 15; the calibration function was calculated by linear regression analysis using the Waters QuanLynx software to:

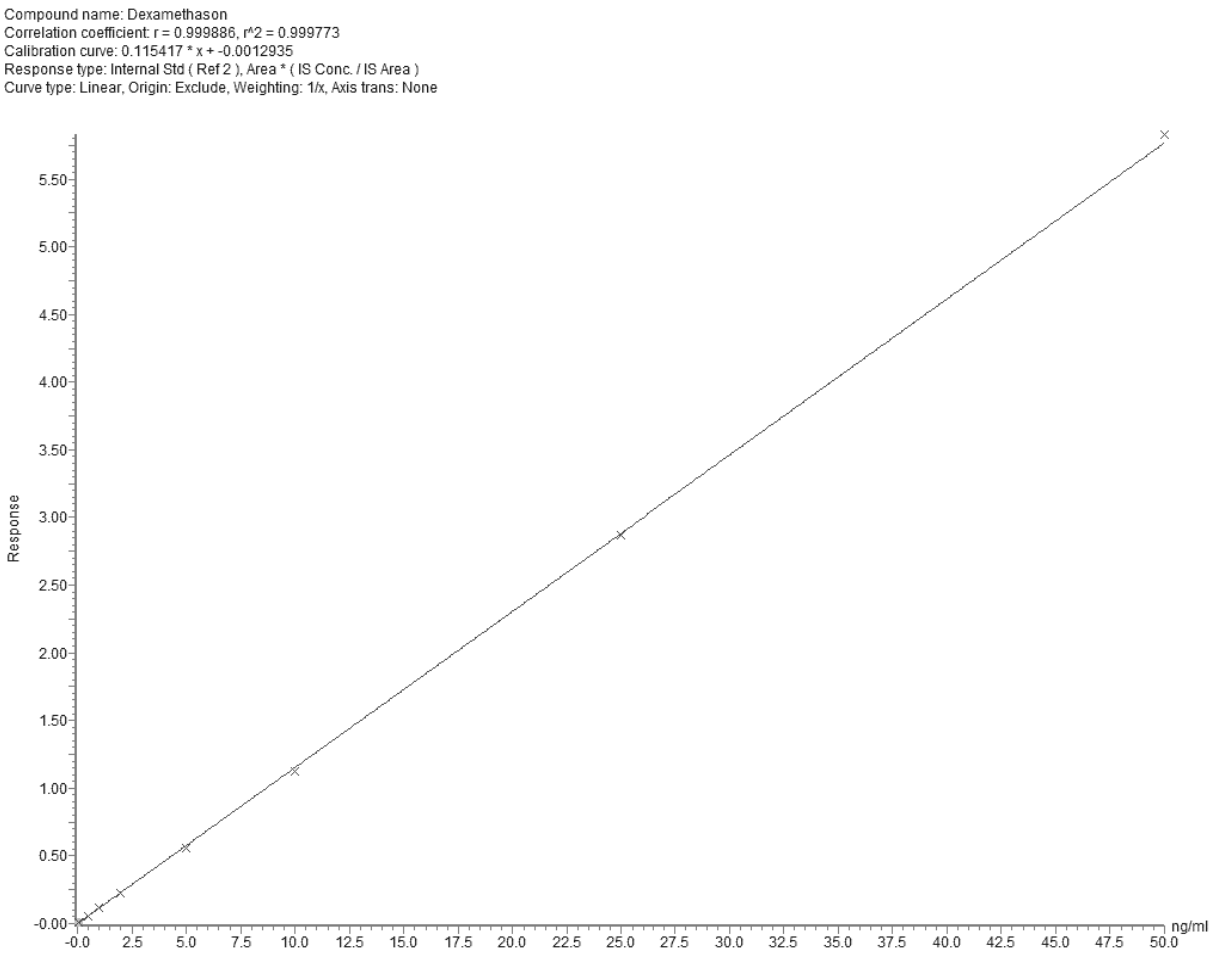
Function: $PAR = 0.1154 \bullet C_{Cal} - 0.0013$ $r^2 = 0.9998$

PAR = Peak area ratio
C_{Cal} = Analyte concentration of the calibration solutions
r^2 = Coefficient of determination

Linearity

Using the linear regression model, the coefficient of determination for Dexamethasone was calculated to 0.9998. As the calculated r^2-values were very close to 1, the linearity of the calibration functions was accepted.

Figure 15: Basic calibration function of Dexamethasone as measured on October 02, 2019.



Results of the analyzed samples

The Dexamethasone concentrations in aqueous test media samples were assessed by chemical analysis, samples of all treatments and the controls were analyzed at test start and at the end of the test (fresh and aged test medium). In the meantime, only the controls, as well as the lowest and highest test concentrations were measured.

The analytical results are listed in Table 54; in addition to samples information, the quantification data, the measured analyte concentrations in water (C_W) and the calculated 'Percent of nominal' values were inserted into the table.

Table 54: Analyzed Dexamethasone concentrations (C_W) and corresponding 'percent of nominal' values (mass transition m/z 393.23 ⇨ m/z 373.17).

Sampling, date and time (2019)	Treatment	Nominal Dexa-methasone conc.	Aging	Dilution factor, DF	LC-MS/MS quantific. data, CLC-MS/MS	Measured analyte conc., CW	Percent of nominal
October 02, day 0	Control	-	Fresh	1	0.019	< LOQ	-
	Conc. 1	1.00 µg/L		1	0.997	1.00	99.7
	Conc. 2	3.20 µg/L		1	3.420	3.42	106.9
	Conc. 3	10.0 µg/L		1	10.639	10.6	106.4
	Conc. 4	32.0 µg/L		1	36.268	36.3	113.3
	Conc. 5	100 µg/L		4	28.077	112	112.3
October 04, day 2	Control	-	Aged	1	0.027	< LOQ	-
	Conc. 1	1.00 µg/L		1	1.038	1.04	103.8
	Conc. 2	3.20 µg/L		1	3.483	3.48	108.8
	Conc. 3	10.0 µg/L		1	10.610	10.6	106.1
	Conc. 4	32.0 µg/L		1	34.256	34.3	107.1
	Conc. 5	100 µg/L		4	25.552	102	102.2
October 09, day 7	Control	-	Fresh	1	0.025	< LOQ	-
	Conc. 1	1.00 µg/L		1	1.023	1.02	102.3
	Conc. 5	100 µg/L		4	27.260	109	109.0
October 11, day 9	Control	-	Aged	1	0.022	< LOQ	-
	Conc. 1	1.00 µg/L		1	1.202	1.20	120.2
	Conc. 5	100 µg/L		4	26.860	107	107.4
October 16, day 14	Control	-	Fresh	1	-	-	-
	Conc. 1	1.00 µg/L		1	1.032	1.03	103.2
	Conc. 5	100 µg/L		4	25.888	104	103.6
	Control	-	Aged	1	0.021	< LOQ	-

Sampling, date and time (2019)	Treatment	Nominal Dexa-methasone conc.	Aging	Dilution factor, DF	LC-MS/MS quantific. data, CLC-MS/MS	Measured analyte conc., CW	Percent of nominal
October 18, day 16	Conc. 1	1.00 µg/L		1	1.174	1.17	117.4
	Conc. 5	100 µg/L		4	25.768	103	103.1
October 23, day 21	Control	-	Fresh	1	0.019	< LOQ	-
	Conc. 1	1.00 µg/L		1	1.115	1.12	111.5
	Conc. 5	100 µg/L		4	26.121	104	104.5
October 25, day 23	Control	-	Aged	1	0.023	< LOQ	-
	Conc. 1	1.00 µg/L		1	1.145	1.15	114.5
	Conc. 5	100 µg/L		4	24.156	96.6	96.6
October 28, day 26	Control	-	Fresh	1	0.040	< LOQ	-
	Conc. 1	1.00 µg/L		1	1.114	1.11	111.4
	Conc. 2	3.20 µg/L		1	3.686	3.69	115.2
	Conc. 3	10.0 µg/L		1	10.336	10.3	103.4
	Conc. 4	32.0 µg/L		1	37.240	37.2	116.4
	Conc. 5	100 µg/L		4	26.230	105	104.9
October 30, day 28	Control	-	Aged	1	0.024	< LOQ	-
	Conc. 1	1.00 µg/L		1	1.077	1.08	107.7
	Conc. 2	3.20 µg/L		1	3.596	3.60	112.4
	Conc. 3	10.0 µg/L		1	10.560	10.6	105.6
	Conc. 4	32.0 µg/L		1	37.618	37.6	117.6
	Conc. 5	100 µg/L		4	25.297	101	101.2

Quality control

The QC samples were processed and measured seven times during the course of the study. For level 1, with a nominal concentration of 2.00 µg/L, the mean recovery was 98.0% with a RSD value of 4.05%, and for level 2, with a nominal concentration of 40.0 µg/L, the mean recovery was 103.0% with a RSD value of 2.29%.

The quality control data show that the measurements were done with high accuracy and precision over the entire run time of the study.

C.2.7 Representative LC-MS/MS chromatograms

Typical LC-MS/MS chromatograms of calibration samples, controls and test media samples are shown in Figure 16 to Figure 23.

Each figure shows four ion chromatograms in stacked windows (top-down):

Ion chromatogram	Mass transition
Dexamethasone, quantification ion	m/z 393.23 ⇨ m/z 373.17
Dexamethasone, qualifier ion	m/z 393.23 ⇨ m/z 355.17
Dexamethasone-d_4 (IS), quantification ion	m/z 397.24 ⇨ m/z 377.17
Dexamethasone-d_4 (IS), qualifier ion	m/z 397.24 ⇨ m/z 359.17

The dashed line in some chromatograms shows the baseline of the integrated peaks executed by automatic integration using the Waters QuanLynx software. However, the grey highlighted part of the chromatographic peak reflects the manually integrated peak area; this corrected peak area was used for quantification of the analyte.

The retention time (t_R) for Dexamethasone was approximately 5.3 min.

Figure 16: Calibration sample CS-1 measured on October 01, 2019; Dexamethasone conc.: 0.08 µg/L.

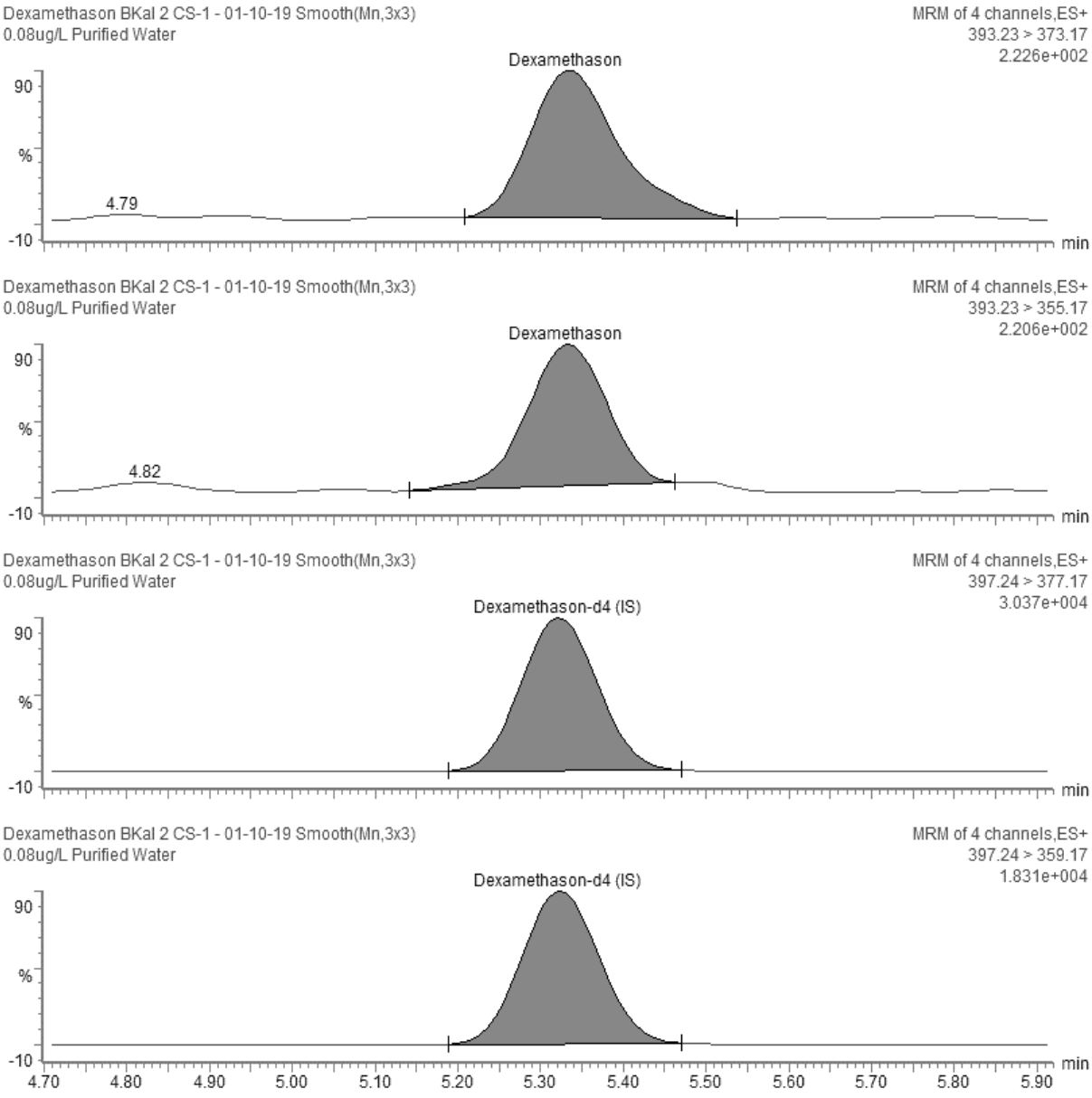


Figure 17: Calibration sample CS-8 measured on October 01, 2019; Dexamethasone conc.: 50.0 µg/L.

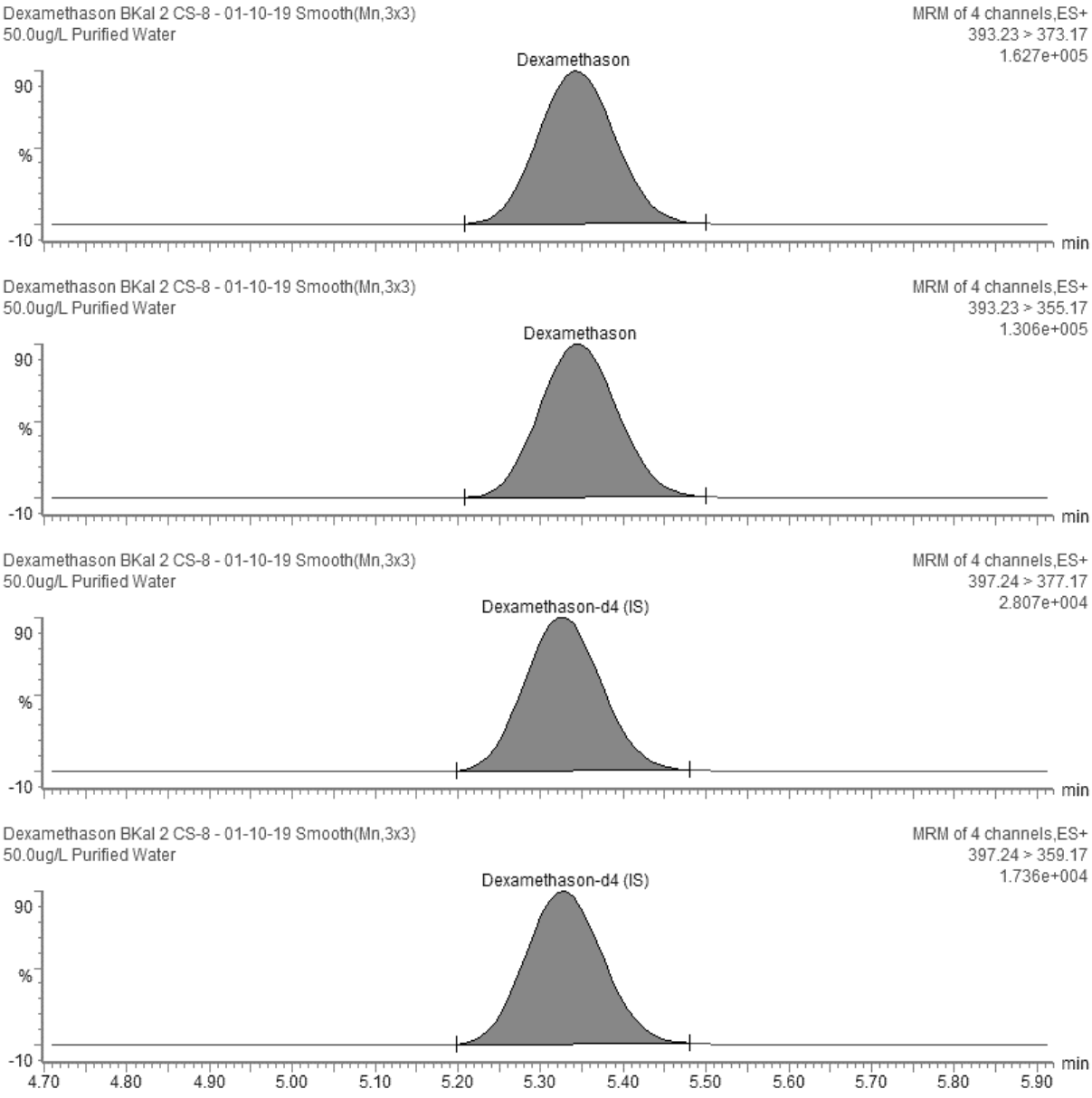


Figure 18: Control sample, fresh test medium, sampling time: test start (October 02, 2019).

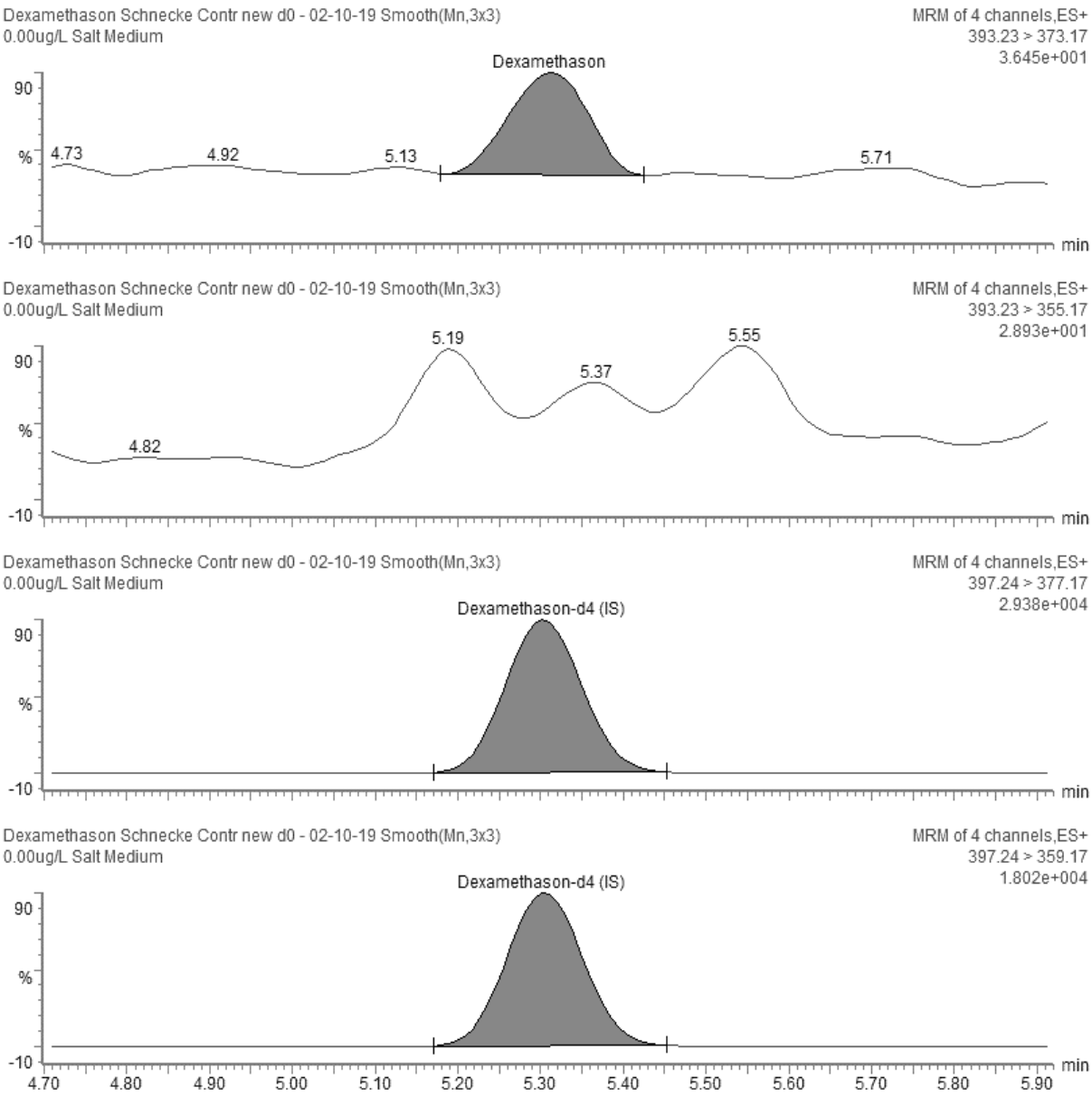


Figure 19: Control sample, aged test medium, sampling time: end of test (October 30, 2019).

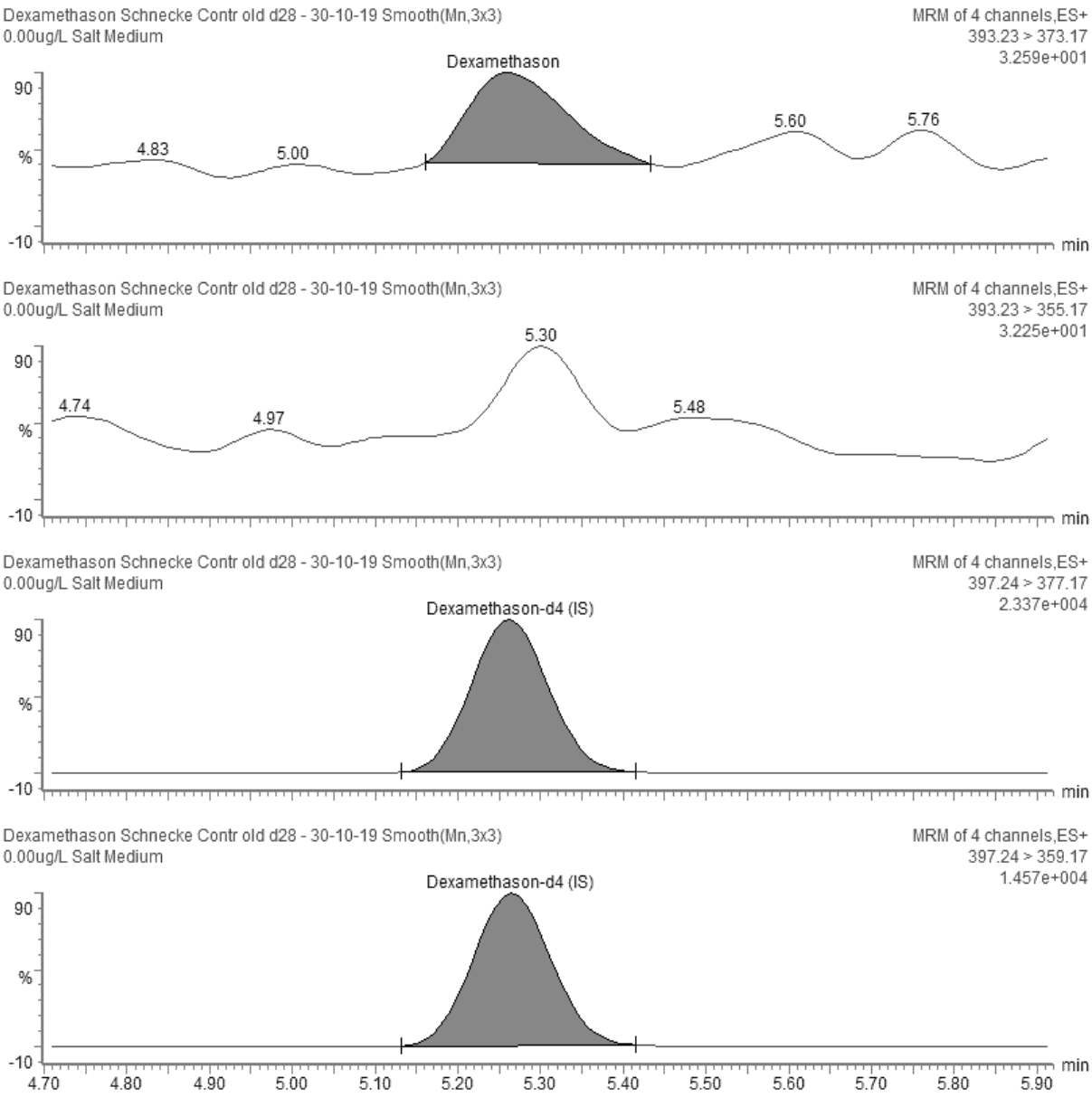


Figure 20: Test media sample, fresh test medium, treatment 1, nominal Dexamethasone conc.: 1.00 µg/L, sampling time: test start (October 02, 2019).

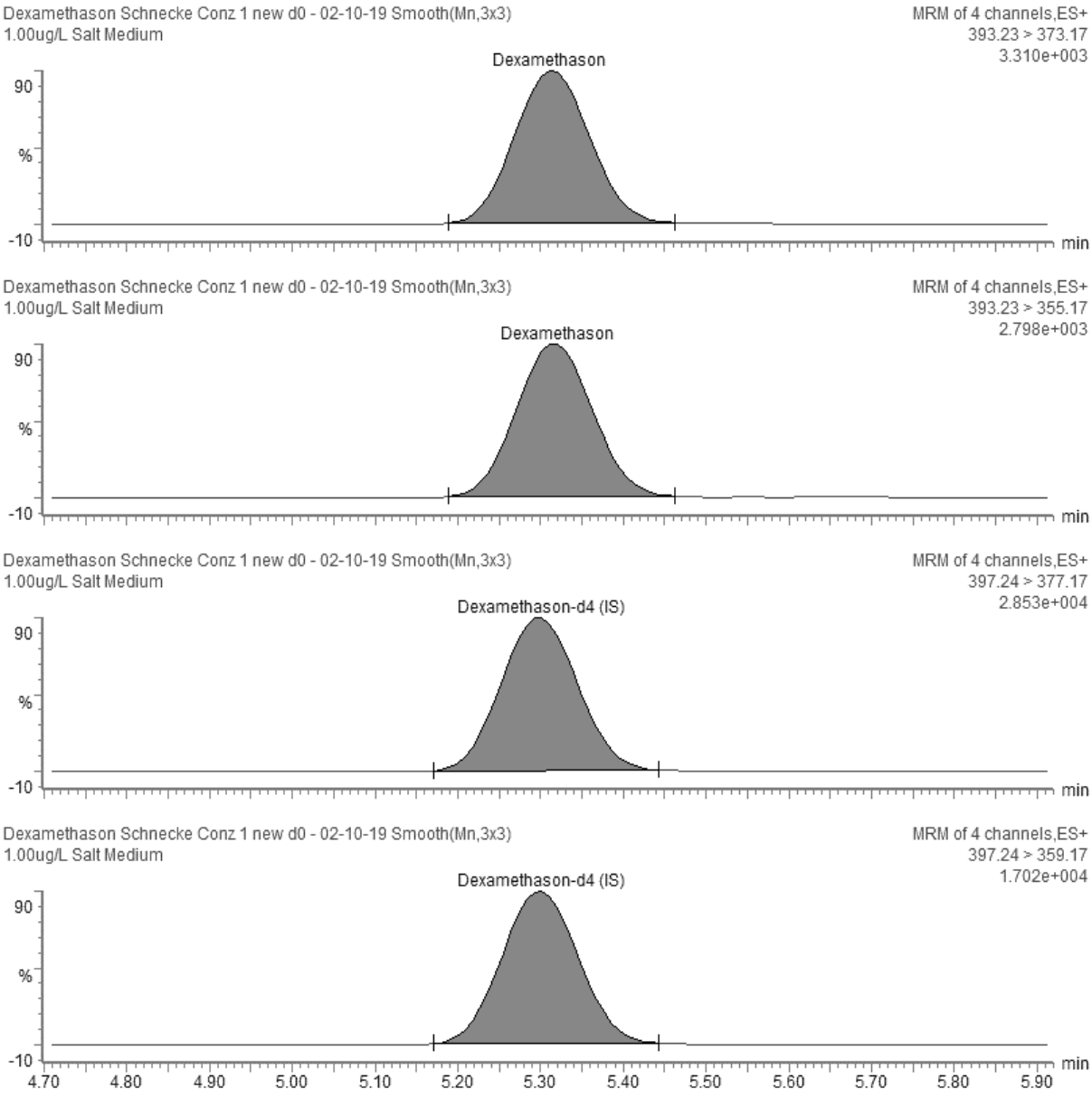


Figure 21: Test media sample, aged test medium, treatment 1, nominal Dexamethasone conc.: 1.00 µg/L, sampling time: end of test (October 30, 2019).

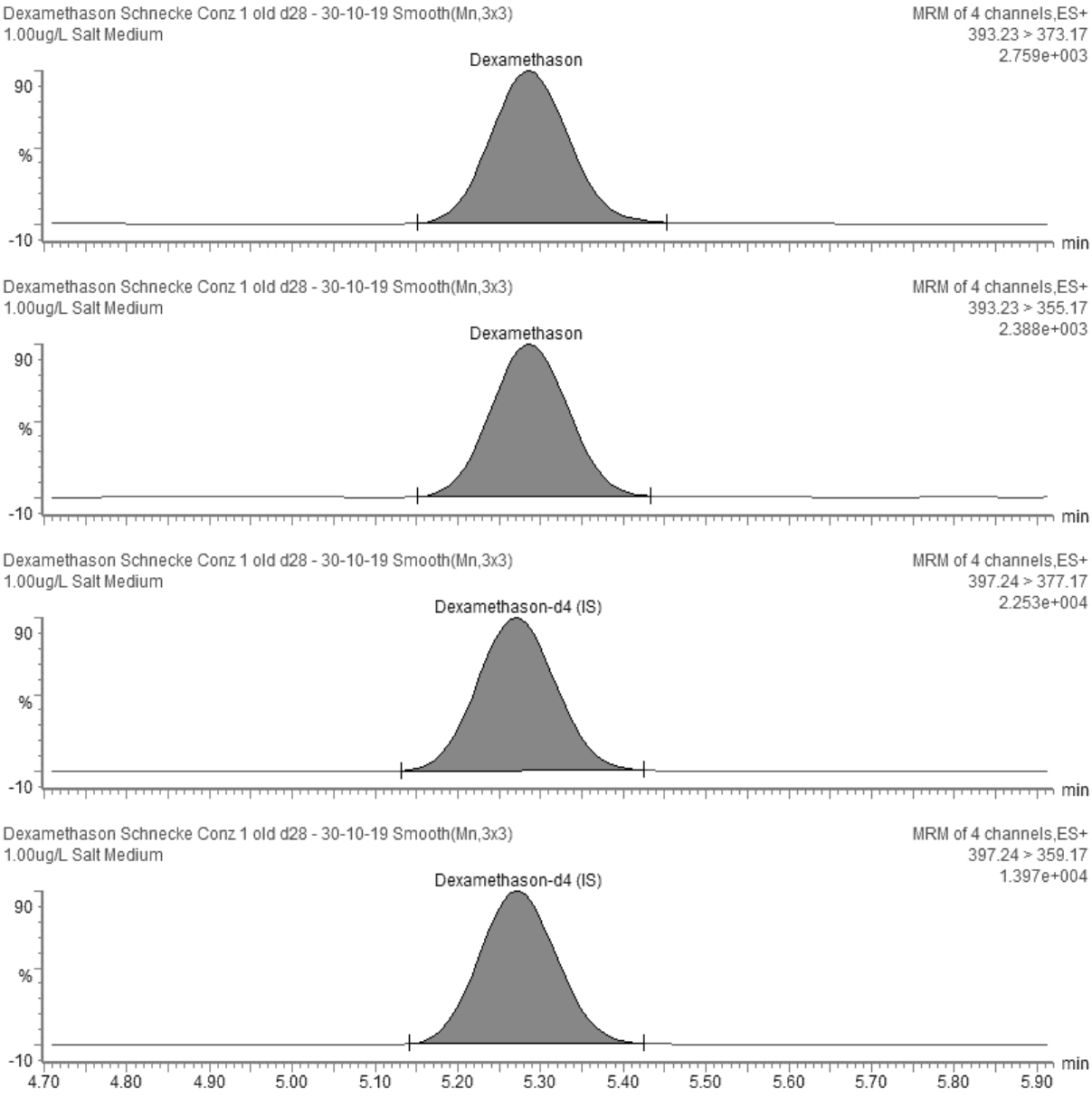


Figure 22: Test media sample, fresh test medium, treatment 5, nominal Dexamethasone conc.: 100 µg/L, sampling time: test start (October 02, 2019).

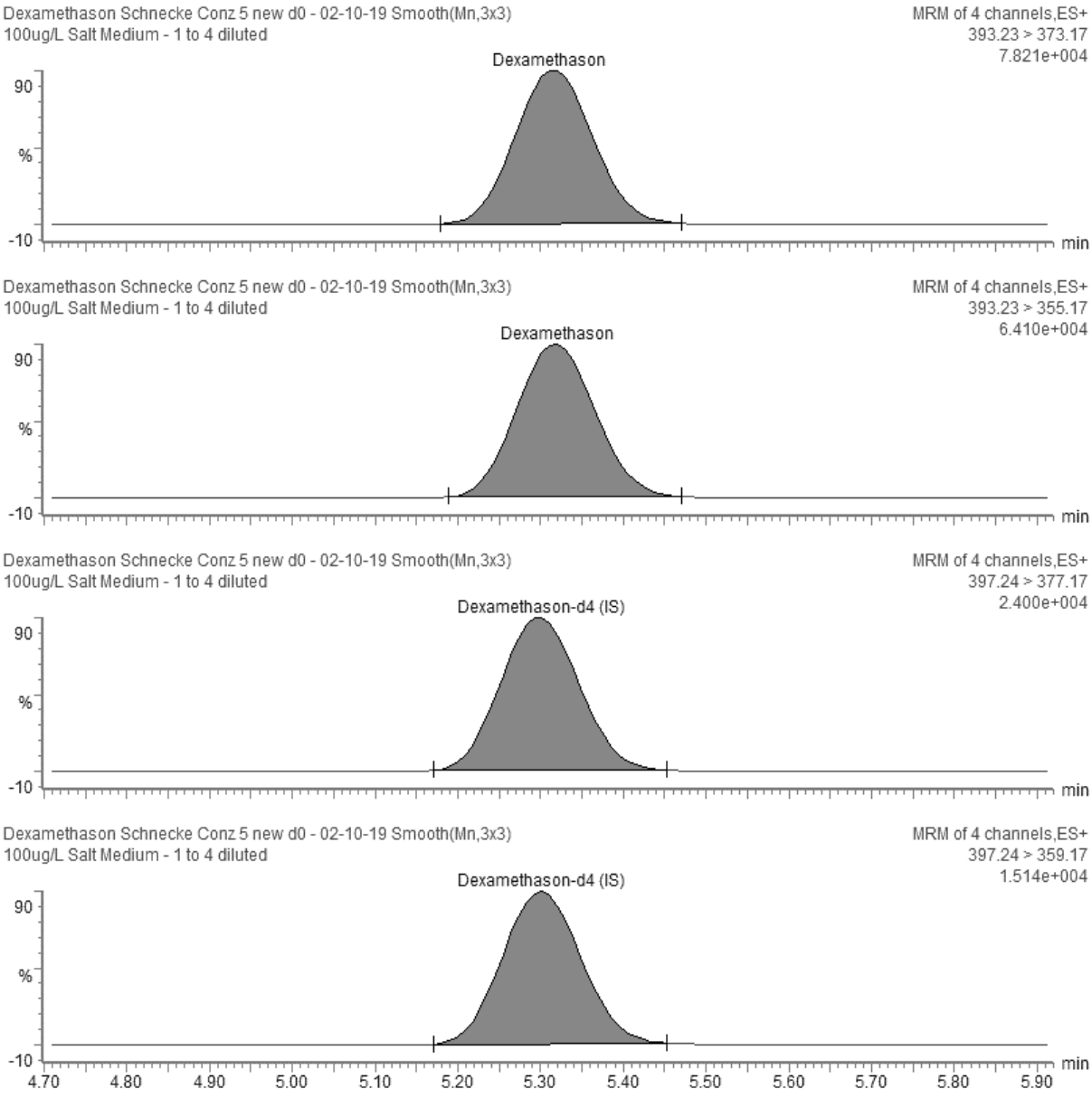
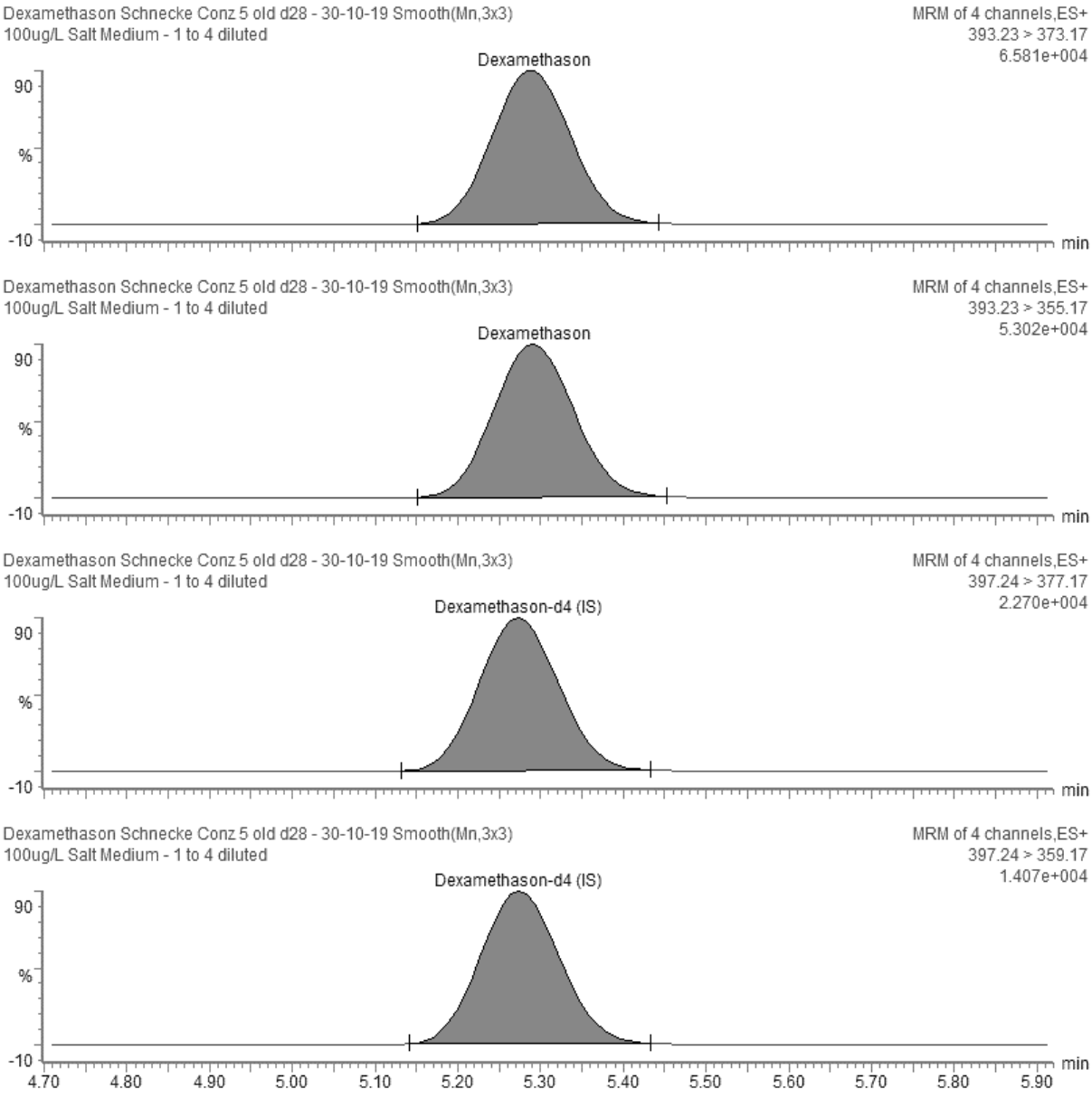


Figure 23: Test media sample, aged test medium, treatment 5, nominal Dexamethasone conc.: 100 µg/L, sampling time: end of test (October 30, 2019).



D Appendix

D.1 Certificate of Analysis

D.1.1 Dienogest

Selleckchem.com

Certificate of Analysis

Print

S1251 Dienogest

Research Area: Endocrinology & Hormones > Estrogen/progestogen Receptor > Dienogest

Name: **Dienogest**
Catalog Number: **S1251**
Batch Number: **S125103**

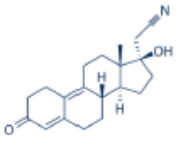
Physical and chemical properties

Molecular Formula: $C_{20}H_{25}NO_2$

Molecular Weight: 311.42
CAS No.: 65928-58-7
Stability:

3 years	-20°C	powder
2 years	-80°C	in solvent

Molecular Structure:



Analytical data

HPLC: 99.68% purity | NMR: Consistent with structure

Toll Free:
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– USA and Canada on

Fax:
+1-832-582-8590

Orders:
+1-832-582-8158
sales@selleckchem.co

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Please provide your
Number in the email.
reply to all email inqu
one business day.

Website:
www.selleckchem.com

1 von 1 31.08.2018

D.1.2 Dexamethasone

Gute Chemie abcr

Certificate of Analysis

Product name:	**Dexamethasone; 98%**
Product no.:	**AB115220**
CAS no.:	**[50-02-2]**
Lot no.:	**1413418**

Test	**Result**
Appearance	white to off-white powder
Purity	98%

Dipl. Ing. Ute Eschrig
Product Safety Manager

Phone +49 (0)721 95061 46
Fax +49 (0)721 95061 33
u.eschrig@abcr.de

Karlsruhe, 19.09.2019

This document is not intended to assure certain properties of products or their suitability for a specific purpose.
This document does not release from industry standard operational control.

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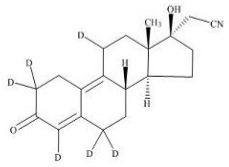
Registered office Karlsruhe
Managing director Dr. Jan Schuricht

D.1.3 Dienogest-d6 (data sheet of the internal standard)

Medical Isotopes Inc.

Providing stable isotope chemicals and research compounds worldwide to universities, laboratories, hospitals, and government agencies.

MY CART

Product:	**Dienogest-d_6**
Catalog Number:	D75937
Unlabeled CAS Number:	65928-58-7
Pricing:	1 mg = $590 (In Stock) Add to Cart
Formula:	$C_{20}H_{19}NO_2D_6$
Molecular Weight:	317.46
Structure:	
Category:	Labeled Reference Standards
MSDS:	Click here.

D.1.4 Dexamethasone (analytical standard)

SIGMA-ALDRICH®

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	DEXAMETHASONE >= 98 % HPLC, powder
Product Number:	D1756
Batch Number:	BCBW7684
Brand:	Sigma
CAS Number:	50-02-2
Formula:	$C_{22}H_{29}FO_5$
Formula Weight:	392.46
Storage Temperature:	2-8 C
Quality Release Date:	23 MAR 2018
Recommended Retest Date:	FEB 2023

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	WHITE TO OFF WHITE	WHITE
APPEARANCE (FORM)	POWDER	POWDER
PURITY (HPLC AREA %)	≥ 98 %	100 %
SOLUBILITY (COLOR)	COLORLESS	COLORLESS
SOLUBILITY (TURBIDITY)	CLEAR	CLEAR
SOLUBILITY (METHOD)	25MG/ML OF METHANOL	25MG/ML OF METHANOL
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
WAVELENGTH (1) (UV)	LAMBDA MAX 239 TO 241 NM	LAMBDA MAX 239 NM
MOLAR ABSORBANCY INDEX (1)	EMM = 15.0 TO 15.5	EMM = 15.1
SOLVENT (UV)	METHANOL	METHANOL

Dr. Reinhold Schwenninger
Quality Assurance
Buchs, Switzerland

D.1.5 Dexamethasone (internal standard)

CERTIFICATE OF ANALYSIS

PRODUCT NAME	Dexamethasone-4,6α,21,21-d_4
PRODUCT NO.	D-5559
BULK LOT NO.	X-473
CAS #	N/A
MOLECULAR FORMULA	$C_{22}H_{25}D_4FO_5$
MOLECULAR WEIGHT	396.49
DATE:	08/23/2019

Stable if stored under recommended conditions (see section 7 of SDS). After three years, the compound should be re-checked for chemical purity before use.

N.M.R.
OK (also 6.5%-d1 deuterated on position C-2)

HPLC
98.2% Chemical purity

Mass Analysis
97.9%-d4

Melting Point
246-248°C

T.L.C.
Single spot

Ying Cao, M.Sc.
Quality Control Manager

C/D/N ISOTOPES INC. - 88 Leacock Street - Pointe-Claire - Quebec - Canada - H9R 1H1
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