UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

TOXIC EFFECTS OF SELECTED NEONICOTINOIDS THROUGH DIFFERENT ORGANISATIONAL LEVELS: *IN VITRO* AND *IN VIVO* STUDIES

DISSERTATION

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"Science... never solves a problem
without creating ten more."

G. B. Shaw

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ABSTRACT

Neonicotinoids are widely applied pesticides due to their higher affinity for insect nicotinic acetylcholine receptors. These compounds are extensively applied to control pest insects in different agricultural crops; however they can also affect non-target organisms (humans or biota). Still a limited number of studies are referring to neonicotinoids in terms of potential hazard for the additive/cumulative effects on human health and to toxic effects of their transformation products on aquatic non-target organisms.

In the scientific literature, data on the effects of IMI, its agricultural products and its transformation products are not sufficient. Due to this fact during this research we wanted to test the toxic effect of imidacloprid-IMI (as pure compound or commercial formulation Confidor 200SL) and its four transformation products (6-chloronicotinic acid-6CNA, desnitro-IMI, olefin-IMI and 5-hydroxy-IMI) applying a battery of acute *in vivo* and *in vitro* bioassays. *In vivo* assays included as model organisms: luminescent bacteria *Vibrio fischeri*, green microalgae *Desmodesmus subspicatus* and amphipod crustacean *Gammarus fossarum*, while *in vitro* assays involved bacteria *Salmonella typhimurium* and sensory neuronal F11 cell line.

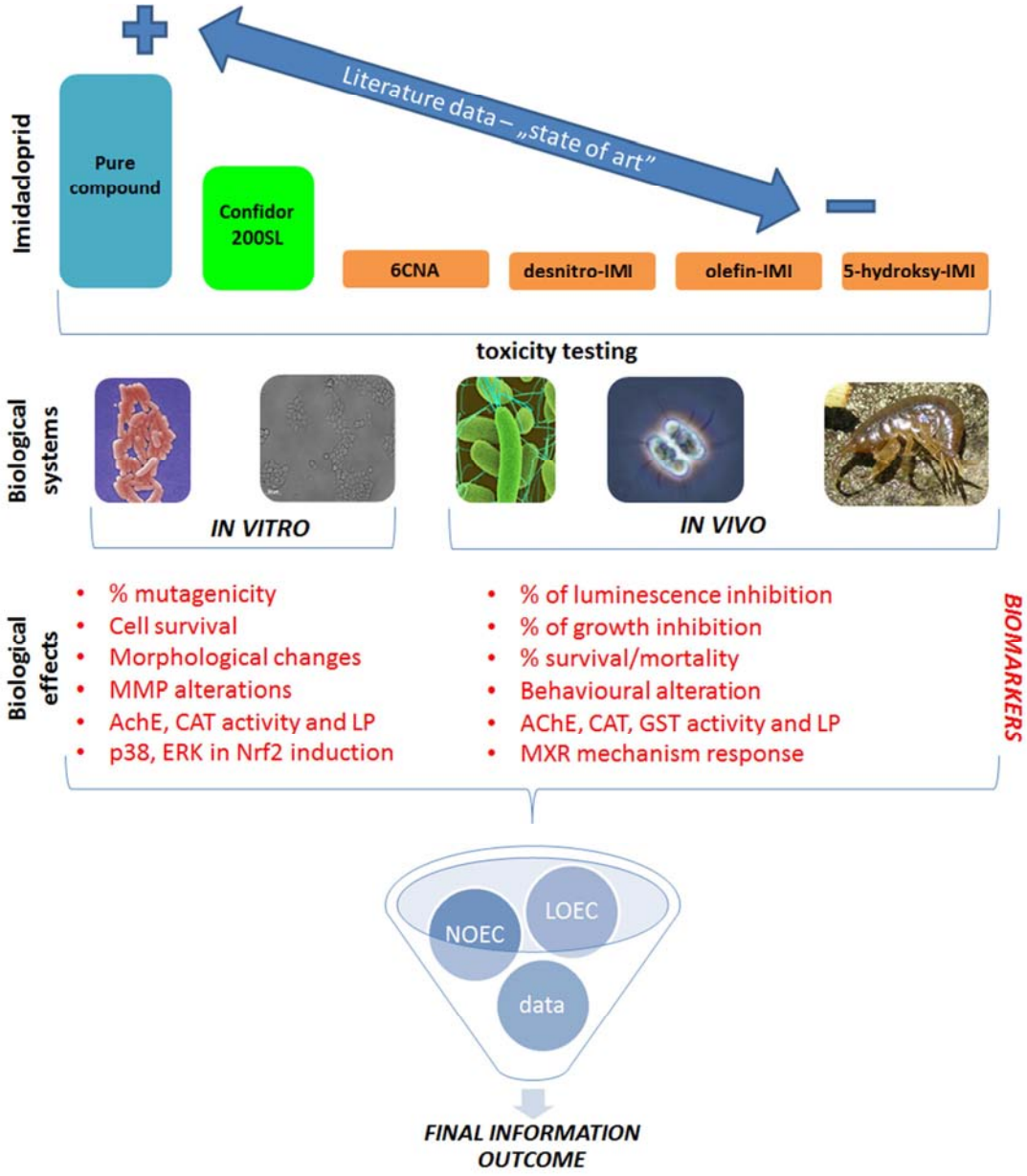
In this work we studied the effects of IMI (1–4000 μM; 0.25–1022.4 mg L^{-1}) on F11 cell line applying a battery of measured parameters. IMI showed significant cytotoxic effects in F11 cells only at concentrations higher than 1000 μM (255.6 mg L^{-1}), confirming its overall low toxicity. More evident reduction of cell survival was in presence of Confidor 200SL, desnitro-IMI, 5-hydroxy-IMI and olefin-IMI compared to pure compound alone. F11 cells exposed for 24 h to IMI (4000 μM; 1022.4 mg L^{-1}) increased the presence of picnotic nuclei, loosed plasma membrane integrity and showed strong degradation of cytoskeleton elements. After 48 h, IMI induced a drastic re-organisation of the cytoskeleton leading to nuclear condensation. F11 cells exposed to IMI (4000 μM; 1022.4 mg L^{-1}) showed early activation of p38- and ERK-mediated intracellular pathways after 30 min, significantly increased lipid peroxidation after 24 and 48 h exposure and slight loss of mitochondrial activity after 15 min at same concentration. Inhibitors of p38 were only moderately sufficient to block IMI cytotoxicity, while anti-oxidant compounds such as vitamin C and E were more protective. These data indicate that oxidative stress induction has a potential

crucial role by which IMI exerts its toxic effects in neuronal cells and suggest caution in potential long-term sensitisation of peripheral sensory neurons even at sub-threshold doses. Results relative to possible mutagenic impact of IMI, Confidor 200SL and 6CNA tested with *S. typhimurium* reverse mutation test did not cause any mutations.

In case of *in vivo* tested models, oxidative stress responses and behavioural changes after 24 h in the crustacean amphipod *G. fossarum* were investigated as well as the growth rate in freshwater algae *D. subspicatus* after 96 h exposure to IMI, its commercial formulation Confidor 200SL and its transformation product 6CNA. Algal growth has shown significant sensitivity to Confidor 200SL and 6CNA acid when compared to IMI. In the case of amphipods, low doses of IMI (102.2 μg L^{-1}) were sufficient to induce lipid peroxidation, while Confidor 200SL induced increased catalase activity (511.3 μg L^{-1}) and peroxidation (255.6 μg L^{-1}). 6CNA altered significantly only antioxidant mechanisms (catalase activity) without changing peroxidation levels. Furthermore, toxicity testing of aqueous solutions for three IMI transformation products (5-hydroxy-IMI, desnitro-IMI and olefin-IMI) was performed with the luminescent bacteria. Different toxic levels of luminescent inhibition were demonstrated also for the IMI products tested on *V. fischeri*.

Given the very different responses of organisms to tested compounds, it would be reasonable to extend the research not only to detect other effects, but also to implement safer use and more efficient removal. These study measurements of different biological effects and responses are helpful to understand the mechanism of IMI or its transformation products-induced oxidative stress. Obtained data demonstrate potential harmful effects of neonicotinoid-based pesticides on non-target organisms. In general, toxicity testing of pesticides should be performed on models at different levels of biological organisation integrated with a multi-biomarker approach providing a complete functional ‘picture’ and better comparison of obtained data.

GRAPHICAL ABSTRACT



POVZETEK

Neonikotinoidi se pogosto uporabljajo kot pesticidi zaradi njihove visoke afinitete vezave na nikotinske holinergične (ali acetilholinske) receptorje žuželk. Neonikotinoidi se pretežno uporabljajo za zatiranje insektov na različnih kmetijskih pridelkih, žal pa lahko vplivajo tudi na netarčne organizme (ljudi, druga živa bitja). Omejeno število študij sicer obravnava neonikotinoide kot potencialno nevarne v smislu aditivnih/kumulativnih učinkov na zdravje ljudi in strupenih učinkov njihovih produktov pretvorbe (npr. intermediatov, vmesnih produktov) na netarčne vodne organizme.

V znanstveni literaturi podatkov o učinkih IMI, njegovih kmetijskih pripravkov ter produktih pretvorbe ni dovolj. Zato smo v sklopu naših raziskav preučevali učinke imidakloprida (IMI), komercialnega pripravka Confidor 200SL ter štirih produktov pretvorbe (6-kloronikotinska kislina-6CNA, deznitro-IMI, olefin-IMI in 5-hidroksi-IMI) z uporabo različnih akutnih *in vivo* kot tudi i*n vitro* bioloških preskusov. *In vivo* preskuse smo izvedli z naslednjimi modelnimi organizmi: luminescenčnimi bakterijami *Vibrio fischeri*, zelenimi algami *Desmodesmus subspicatus* ter raki postranicami *Gammarus fossarum*, medtem ko so *in vitro* biološki preskusi vključevali bakterije *Salmonella typhimurium* ter senzorske nevronske celice F11 .

Za ugotavljanje učinkov IMI-ja (1–4000 μM; 0.25–1022.4 mg L^{-1}) smo uporabili F11 celično linijo, pri kateri smo merili serijo različnih parametrov. Rezultati so pokazali citotoksične učinke le pri koncentracijah IMI, višjih od 1000 μM (255.6 mg L^{-1}), s čimer smo potrdili njegovo poznano nizko toksičnost. Opaznejše zmanjšanje preživetja celic smo zabeležili v prisotnosti Confidorja 200SL, deznitro-IMI-ja, 5-hidroksi-IMI-ja ter olefin-IMI-ja v primerjavi z imidaklopridom. Ko smo F11 celice izpostavili IMI-ju za 24 ur (4000 μM; 1022.4 mg L^{-1}) je prišlo do porasta piknotičnih jeder, izgube integritete plazemske membrane ter znatnega propada citoskeletnih elementov. Po 48 h je IMI induciral intenzivno reorganizacijo citoskeleta v smeri kondenzacije jeder. F11 celice, izpostavljene IMI-ju (4000 μM; 1022.4 mg L^{-1}) so po 30 min izkazovale zgodnjo aktivacijo p38- in ERK intracelularnih poti, po 24 in 48 h izpostavitvi pa značilno povišano stopnjo lipidne peroksidacije ter rahlo znižanje mitohondrijske aktivnosti po 15 min. Inhibitorji p38

so le delno blokirali IMI-jevo citotoksičnost, medtem ko so druge antioksidativne spojine, kot sta vitamina C in E, delovale bolj zaščitno. Ti rezultati nakazujejo, da ima indukcija oksidativenga stresa pomembno vlogo, s čimer IMI strupeno deluje na nevronske celice. Zato je potrebna previdnost v primeru dolgotrajne preobčutljivosti perifernih senzornih nevronov že pri koncentracijah, ki so pod pragom strupenosti. Rezultati testiranj z uporabo *S. typhimurium*, vezanih na možne mutagene učinke IMI-ja, Confidorja 200SL ter 6CNA, niso pokazali nikakršnih mutacij.

V primeru *in vivo* bioloških testov, smo v rakih postranicah *G. fossarum* preučevali odzive, vezane na oksidativni stres ter spremembe obnašanja po 24 h izpostavitvi, v sladkovodnih algah *D. subspicatus* pa hitrost rasti po 96 h izpostavitvi IMI-ju, komercialnemu pripravku Confidorju 200SL ter pretvorbenemu produktu 6CNA. Rast alg je bila manjša pri izpostavitvi Confidorju 200SL in 6CNA v primerjavi z IMI. V primeru rakov so le nizke koncentracije IMI-ja (102.2 $\mu g\ L^{-1}$) inducirale lipidno peroksidacijo, medtem ko je Confidor 200SL induciral tako katalazno aktivnost (511.3 $\mu g\ L^{-1}$) kot peroksidacijo (255.6 $\mu g\ L^{-1}$). 6CNA je vplivala na antioksidativni mehanizem (katalazna aktivnost) medtem ko na peroksidacijo ni vplivala. Nadaljnji poskusi, pri katerih smo luminescenčne bakterije izpostavili vodnim raztopinam treh produktov pretvorbe IMI-ja (5-hidroksi-IMI, deznitro-IMI in olefin-IMI) so pokazali različno strupenost v primerjavi z IMI-om.

Glede na zelo različne odzive organizmov na testirane spojine, bi bilo smisleno raziskave razširiti ne le na ugotavljanje drugih učinkov, pač pa tudi na varnejšo uporabo ter učinkovitejše odstranjevanje. Omenjena raziskava z meritvami različnih učinkov in odzivov v bioloških preskusih omogoča razumevanje mehanizma oksidativnega stresa, povzročenega zaradi delovanja IMI-a ter njegovih produktov pretvorbe. Dobljeni rezultati namreč kažejo na morebitne škodljive učinke neonikotinoidnih pesticidov na netarčne organizme. Na splošno pa lahko trdimo, da moramo strupenostne teste s pesticidi izvesti na modelnih sistemih na različnih nivojih biološke organizacije z uporabo večjega števila biomarkerjev. Na ta način zagotovimo učinkovitejšo primerjavo podatkov ter popolnejšo sliko delovanja preiskovanega pesticida in razumevanje pri katerih koncentracijah ima substanca kvarne učinke.

GRAFIČNI POVZETEK

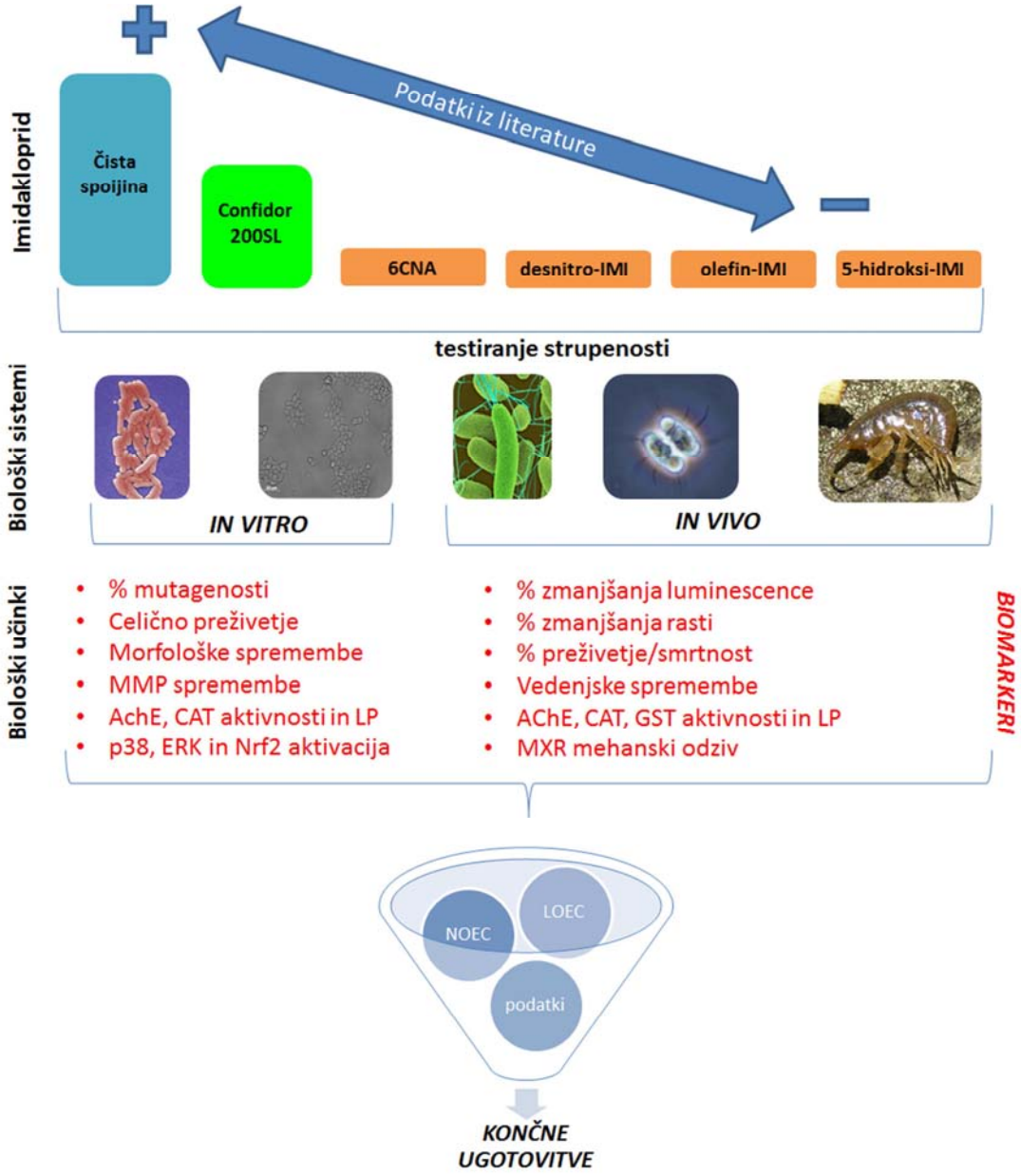


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Figure 35: Values of MTT assay in F11 cells after exposure to IMI, Confidor 200SL and 6CNA for 24 h (A) and 48 h (B); data presented as percentage of control shown as dashed line (n=6). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

Figure 36: Values of MTT assay in F11 cells after exposure to IMI, desnitro-IMI, 5-hydroxy-IMI and olefin-IMI for 24 h (A) and 48 h (B); data presented as percentage of control shown as dashed line (n=6). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

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Figure 46: Whole-body GST activity (nmol/min/mg protein) of *G. fossarum* measured after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75% of all

readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). (n = 10). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

Figure 47: Whole-body LP of *G. fossarum* (expressed in absorbance units of TBARS products) measured after 24 h of exposure IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). (n=10). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

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Figure 49: Schematic diagram describing the possible cytotoxic model of IMI and its activated molecular pathways in human neuroblastoma F11 cells. (YNM, 2010, with slight modifications).

ABBREVIATIONS AND SYMBOLS

a. i.	active ingredient
ACh	acetylcholine
AChE	acetylcholinesterase
nAChe	nicotinic acetylcholine receptor
ATP	adenosine triphosphate
AU	absorbance unit/ arbitrary unit
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
BTH	butylatedhydroxytoulene
CAT	catalase
CCD	colony collapse disorder
CDNB	1-chloro-2,4-dinitrobenzene
CGRP	calcitonin gene-related peptide
DMSO	dimethyl sulfoxide
DTNB	5,5 dithiobis-2-nitrobenzoic acid
DMEM	Dulbecco's modified Eagle medium
$EC_{50\ (20)}$	median (50 %; 20%) effective concentration
ECPA	European Crop Protection Association
ECVAM	European Centre for Validation of Alternative Methods
EFSA	European Food Safety Authority
em.	Emission
ERA	Environmental Risk Assessment
ETS	electron transport system
ex.	Excitation
FAO	Food and Agricultural Organisation
FBS	foetal bovine serum
GDP	gross domestic product
GP_x ($GSH\text{-}P_x$)	glutathione peroxidase
GSH	Glutathione
GST	glutathione-*S*-transferase
G6PD	glucose-6-phosphate dehydrogenase

HPLC-DAD	high performance liquid chromatography – diode array detector
HQ	hazard quotient
IMI	Imidacloprid
IC_{50}	median (50 %) inhibition concentration
IF	Immunofluorescence
IPM	Integrated Pest Management
LC_{50}	median (50 %) lethal concentration
LC-MS	liquid chromatography-mass spectrometry
LD_{50}	median (50 %) lethal dose
LGIC	ligand-gated ion channel
LO(A)EC(L)	lowest observed (adverse) effect concentration (level)
LP	lipid peroxidation
MAPK	mitogen activated kinase
MDA	Malondialdehyde
MMS	methyl methanesulphonate
MRL	maximum residue level
MRP	multidrug resistance protein
MXR	multixenobiotic resistance
NAC	*N*-acetyl-*L*-cystein
NADP	β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate
NGF	neuronal growth factor
NMP	*N*-methylpirrolidone
NO(A)EC(L)	no observed (adverse) effect concentration (level)
Nrf2	nuclear factor (erythroid-derived 2)-like 2
NRC	National Research Council
PBS	phosphate buffered saline
PEC	predicted environmental concentration
PLL	poly-*L*-lysine hydrochloride
PKC	protein kinase C
ppb	part per billion
ppm	part per million

PPP	plant protective products
ROS	reactive oxygen species
RNS	reactive nitrogen species
RQ	risk quotient
RT	room temperature
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TN	total nitrogen
TOC	total organic carbon
TP	transformation product
US EPA	US Environmental Protection Agency
WHO	World Health Organisation
6CNA	6-chloronicotinic acid

INTRODUCTION

1. INTRODUCTION

Pesticides are the pollutants increasingly present in the surrounding environment, which are often persistent and can be bioaccumulated through the biological chains such as soil-plant-food or water-aquatic organism-food (Preston, 2002). The usage of high amount of pesticides in environment represents a possible risk for biota and human health due to their potential toxic action. Pesticide substances are **biologically active** and must be tested to ensure that their use will not give rise to any unacceptable risks to **non-target organisms** (i.e. humans, animals and plants) or to the environment. As part of these testing, pesticides are fully investigated for potential acute, sub-chronic and chronic toxic effects with the use of different **biological systems** and with the measurement of selected **biomarkers** or conventional **endpoints**.

1.1. Pesticides in general

As the Food and Agriculture Organisation of the United Nations (FAO) defined, pesticide is any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest, including vectors of human or animal disease or weed which can cause harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs. Pesticides can be classified according to their **target**, their **mode or period of action**, or their **chemistry**. They may be chemical substances, biological agents (such as viruses or bacteria), antimicrobials, disinfectants or devices used against any pest (Saravi and Shokrzadeh, 2011). Mainly, agricultural pesticides are divided into five categories based on the targeted pest and include: insecticides, herbicides, fungicides, rodenticides, and fumigants (Saravi and Shokrzadeh, 2011). Recently, the European Food Safety Authority (EFSA) tried to replace the expression ‘pesticide’ due to its negative implications, with the new term-‘plant protective product’ (PPP).

Pesticides are a highly diverse group of compounds and present one of the most important groups of chemical stressors in the environment (Hanazato, 2001; Liess and Ohe, 2005). As an alternative to first generation pesticides, modern pesticides have been designed to avoid some of the deleterious effects of these pesticides that had a long residence time in the environment and accumulated in the food chain (Meleiro Porto *et al*., 2011). Newer classes of highly selective, systemic and single mode activity pesticides were introduced in the early 1990s and promised to address pest more specifically. Over the past twenty years, a class of systemic insecticides called **neonicotinoids** gained increasing interest in the agricultural sector. The term **'neonicotinoid pesticides'** comprises a group of several different **insecticides**, but it is usually used to indicate the four that are most widely applied: imidacloprid, thiacloprid, clothianidin and thiamethoxam. One of these representatives**, imidacloprid (IMI)**, was the main focus of this research.

In the last decades, the world has known for a continuous growth of pesticide usage, both in number of chemicals and quantities sprayed over the agricultural fields (Carvalho, 2006). According to the data supplied by European Crop Protection Association (ECPA), the total amount of PPP used in the European Union (reported as tonnes of active ingredient (a. i.) for the main PPP categories) increased steadily in the 1990s, stabilised in the late '90s and these data have not altered significantly between 1992 and 2003 (Fig. 1A). Especially, is important to notice that the use of insecticides more than doubled between 1992 and 2000, with only a slight decrease from 2000 to 2003 (Fig. 1B) (EUROSTAT, 2007).

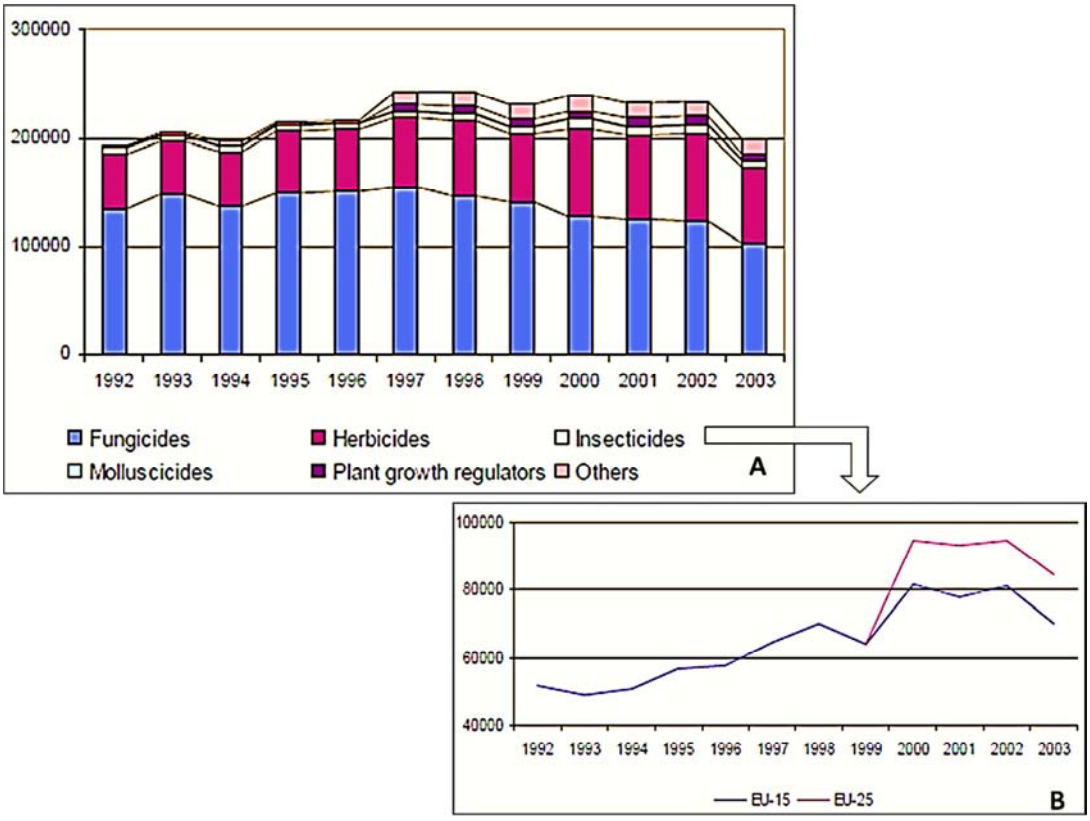


Figure 1: (A) Use and composition of PPP in 15 Member States of the European Union (EU-15), 1992-2003 (y-axis; in tonnes of a. i.) (B) Use of PPP-insecticides in all Member States of European Union (EU-25) and in EU-15, 1992-2003 (y-axis; in tonnes of a. i.). (EUROSTAT, 2007).

The Community regulatory framework concerning pesticides focuses particularly on the placing on the market and the end of the life cycle of such products. The most relevant legislative measures concerning PPPs are:

1) Directive 91/414/EEC on the placing of PPPs on the market.
2) Regulation (EC) No. 396/2005 on maximum residue levels of pesticides in food and feed.
3) Water Framework Directive (WFD) 2000/60/EC.

Lately, the EC Directive 91/414/EEC has been replaced by EC Directive 1107/2009. The new legislation published in December 2009 which ispart of the European Union Thematic Strategy on Pesticides and is composed of four elements:

1) Plant Protection Products Regulation 1107/2009
2) Sustainable Use Directive (SUD) 2009/128/EC
3) Machinery Directive 2009/127/EC – which sets out standards for new equipment
4) Statistics Regulation 1185/2009 – the key elements of this regulation are the provision of annual sales data and the provision of data every five years on usage on crops and the pesticides applied.

These above-mentioned legislations are also applied in Slovenia that is one of the Member States of the EU. In comparison to other Central and Eastern European countries, the macroeconomic importance of agriculture in Slovenia is relatively low. Share of agriculture, hunting and forestry in its gross domestic product (GDP) has been decreasing in the last decade (Slabe, 2001). The PPP market in Slovenia is dominated by herbicides and fungicides in comparable proportions (Fig. 2). Over the last years, total pesticide products use has been increasing. Average use of pesticide products in 2000 was estimated at 3.1 kg ha^{-1} of agriculture land (GIS, 2003).

The use of **neonicotinoids** has been approved in the EU and they are currently in use. IMI, thiamethoxam, acetamiprid and thiacloprid are registered also on the Slovenian market. However, since May 2008, the insecticides thiamethoxam, clothianidin and IMI were banned for treatment of corn seeds (*Zea mays*), sugar beet seeds (*Beta vulgaris*) and oilseed rape seeds (*Brassica napus*) (The Official Gazette of the Republic of Slovenia No. 50/2008). Despite all the precautions taken in order to avoid the poisoning of bees, a large number of bee's colonies died in the eastern part of Slovenia during April 2011 (Pomor čebel v Pomurju dobiva nove razseznosti, 2011). Due to these repetitive negative events, the Phytosanitary Administration of Slovenia banned completely clothianidin, IMI and thiamethoxam for any seed treatment, also other EU member states such as France, Germany and Italy imposed similar bans for IMI and clothianidin. Moreover, clothianidin is completely banned in Slovenia for any agricultural use (The Official Gazette of the Republic of Slovenia No. 31/2011). It was estimated that in Slovenia approximately 2,400 kg of neonicotinoids was sold in the year 2004 (Phytosanitary Administration of the Republic of Slovenia, personal communication, 2005; Žabar, 2012).

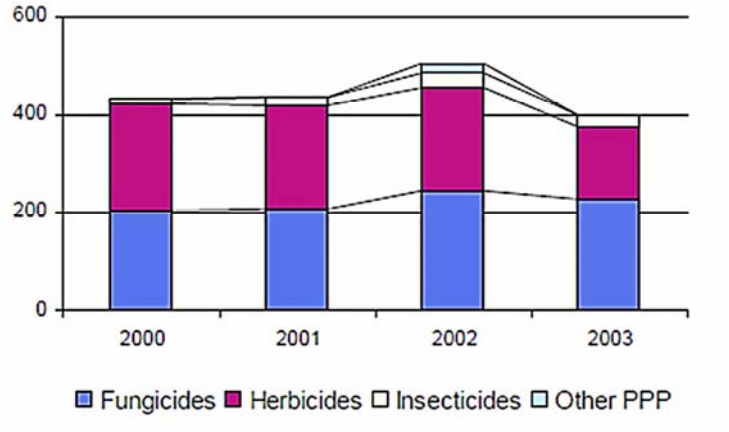


Figure 2: Use and composition of PPP in Slovenia, 2000-2003 (y-axis; in tonnes of a.i.) (EUROSTAT, 2007).

1.2. Neonicotinoids – Imidacloprid

Neonicotinoids are a group of insecticides derived from nicotine isolated from the tobacco plant (*Nicotiana tabaccum*) which presents insecticidal activity and has been used extensively as natural insecticide. The developing road from nicotine to neonicotinoids was long and complicated. Neonicotinoids effective control of insect pests and helminthic parasites has been achieved by targeting invertebrate (insect) nAChRs (Matsuda *et al*., 2005; Tomizawa and Casida, 2003; 2005). The major commercial insecticides targeting insect nAChRs were not derived from natural products but rather from the discovery of synthetic nitromethylene heterocyles (Kagabu, 1997). Introduction of the 6-chloro-3-pyridylmethyl and nitroimine moieties led to the development of the first type of nicotinic insecticide called imidacloprid (Kagabu, 1997).

Imidacloprid (IMI) is one of the major representatives of the new generation of neonicotinoid insecticides. It was patented for the first time in 1985 by Bayer and was placed on the market in 1991. Today it has been made commercially available by Bayer AG and Nihon Tokushu Noyaku Seizo KK. It is a nicotine derived compound (neonicotinoid) with a large potential distribution due to its agonistic action on insect nAChRs and its selective toxicity to insects over vertebrates (Tomizawa and Casida, 2003). IMI [1-[(6-chloro-3 pyridynil) methyl]-*N*-nitro-2-imidazolidinimine] has the molecular formula $C_9H_{10}ClN_5O_2$ (Fig. 3), with a molecular weight of 255.7 g mol^{-1}. In appearance, it consists of colourless crystals.

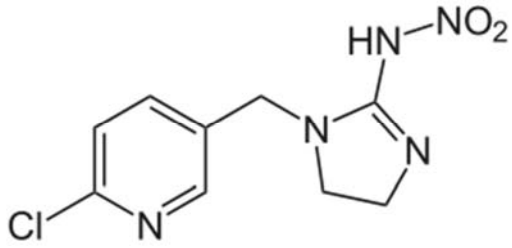


Figure 3: Chemical structure of imidacloprid.

This component is a relatively new pest control substance, which is having the fastest growing sales worldwide (Tomizawa and Casida, 2005) and is generating increasing concern on its possible impacts on natural ecosystems (Matsuda *et al*., 2001; Jemec *et al*., 2007). Elbert *et al*. (2008) pointed out remarkable data revealing the turnover toward insecticidal seed treatment. By the year 2005 seed coating developed into a €535 million market, with a 77 % share for neonicotinoid insecticides.

IMI's physicochemical properties render it useful for a wide range of application techniques, including foliar, seed treatment, soil drench, and stem application (Millar and Denholm, 2007) (Table 1). It is a versatile, broad-spectrum, systemic insecticide with activity against sucking insects (e.g. aphids, leafhoppers, whiteflies, and termites), several species of *Coleoptera, Diptera, Lepidoptera* and parasites on a different types of crops (Tomlin, 1994; Tomizawa and Casida, 2005). In addition, it is also applied as veterinary medicine against parasites and fleas in dogs and cats. Other important neonicotinoids are acetamiprid, clothianidin, dinotefuran, thiacloprid and thiamethoxam.

Commercial formulations of IMI used for the control of different pest insects are available in a very large number (e.g. Admire, Merit, Confidor 200SL, Provado and Gaucho) (PMRA, 2005). Formulations of IMI are present as: a slurry or flowable concentrate for seed treatments, granule, wettable powder, soluble concentrate, and suspension concentrate, water dispersible granules, and dustable powder (Tomlin, 2004). To make a pure pesticide molecule applicable and highly functional, a. i. are combined with solvents or surfactants to accomplish an effective action of these formulations in agricultural applications. These supplementary substances often represent the highest proportion in pesticide marketed mixtures and include co-formulants (e.g. dimethyl sulfoxide (DMSO), *N*-methylpyrrolidone (NMP), propylene carbonate and other solvents) that could modify IMI's toxicity and bioavailability. Formulations of IMI include also other chemicals such as crystalline quartz silica and naphthalene chemicals which have also associated toxicological characteristics (Cox, 2001). Even a minor concern regarding the toxicity of these compounds and their possible synergistic effect with IMI or other ingredients should be continuously considered (Tobiassen *et al*., 2003; Surgan, 2005). It is important to

notice that the commercial formulations are the ones applied in the environment with relevant soil (50 g ha^{-1} to 320 g ha^{-1}) and foliar concentrations (73 mg L^{-1} to 150 mg L^{-1}). Recent evaluation of the data relative to different formulations noted high levels of IMI in leaves and in blossoms of treated plants, and increases in residue levels over time (CDPR, 2011). Data indicated that the use of these IMI formulations on annual basis may be at the end cumulative. Due to recent findings certain commercial products within the class of neonicotinoids (containing a. i. IMI) were placed under re-evaluation and need further studies (CDPR, 2011).

Even if IMI has been in use for a relatively short period of time compared to other common pesticides, it is considered to being used in the largest quantity worldwide of all insecticides (Cox, 2001; Ware and Whitacre, 2004). Comparative toxicity studies indicate IMI's adverse effects to some aquatic invertebrates and have noticed high species-specific response to IMI, which suggests that IMI toxicity data may not be generalised (Jemec *et al*., 2007). In addition, IMI can be applied at very low rates and during whole year.

In the present research, IMI - as **pure compound** and as **commercial formulation Confidor 200SL** was selected for further studies and investigated for potential toxicity to different non-target organisms (Fig. 4).

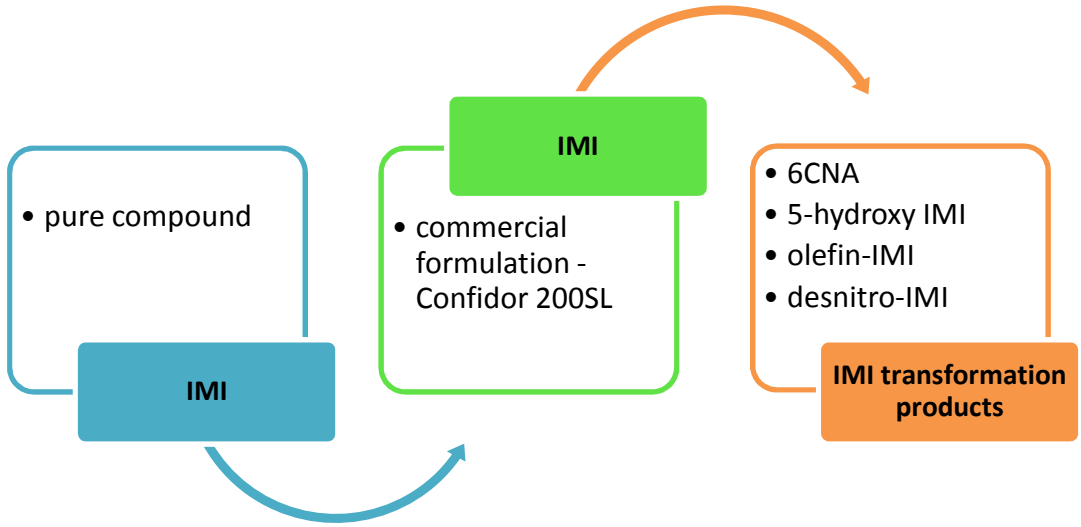


Figure 4: Schematic diagram of selected compounds which were tested on different biological systems during this study.

Table 1: Selected properties of IMI.

Imidacloprid

Physico-chemical properties		*Ref. No.*
CAS number	138261-41-3	(1)
Water solubility at 20°C (mg L^{-1})	(a) 510 (b) 610	(a) (2) (b) (1, 3)
K_{oc} (soil organic carbon–water partitioning coefficient)	(a) 210 (20 °C) (b) 249-268 (c) 109-411 (20°C)	(a) (4) (b) (5) (c) (3)
Log K_{ow} (octanol-water partition coefficient)	(a) 0.57 (b) 0.92	(a) (1) (b) (4)
Average application rates: soil (g ha^{-1}) and foliar (mg L^{-1}) applications	(a) 50-320 (b) 73-150	(a) (6) (b) (7)
Environmental fate		***Ref. No.***
Detected aquatic concentrations (µg L^{-1})	(a) 1 (surface water, Florida, USA) (b) 6.7 (ground water, Florida, USA) (c) 14 (Lake Wales Ridge, USA) (d) 300 (surface water, Denmark)	(a) (8) (b) (9) (c) (10) (d) (11)
Estimated aquatic concentrations (µg L^{-1})	(a) 36.04 (acute surface water exposure); 17.24 (chronic surface water exposure) (b) 22 (accidental direct spray in a pond or stream); 1.8-7.3 mg L^{-1} (accidental spill in a small pond)	(a) (9) (b) (12)
Aqueous photolysis DT_{50}	(a) 3 h (simulated sunlight, 30°C) (b) >1 h (simulated sunlight, 24 °C)	(a) (13) (b) (9)
Hydrolysis DT_{50} (days)	(a) > 30 (b) 355 (at pH 9); stable at pH 5 and 7 (c) 36.3 at pH 4; 41.6 at pH 7	(a) (9) (b) (14) (c) (15)
Soil photolysis DT_{50} (days)	38.9	(9)
Soil anaerobic DT_{50} (days)	27.1	(9)
Soil aerobic DT_{50} (days)	(a) 156 (b) 997	(a) (3) (b) (9)
Field dissipation DT_{50} (days)	(a) 26.5 – 229 (b) 83 - more than 365 (c) 7 (corn); 12 (sandy loam); turf grass (61-107) (d) 96 (bare soil)	(a) (9) (b) (16) (c) (17) (d) (3)

References: *(1) Tomlin, 2004; (2) Yen and Wendt, 1993; (3) Krohn and Hellpointer, 2002; (4) Nemeth-Konda* et al.*, 2002; (5) Oi, 1999; (6) PMRA, 2005; (7) www.bayercropscience.com; (8) Jemec* et al.*, 2007; (9) Fossen, 2006; (10) US Geological Survey, 2003; (11) Tennekes, 2010; (12) SERA, 2005; (13) Kagabu and Medej, 1995; (14) US EPA, 2005; (15) Sarkar* et al.*, 1999; (16) Sabbagh* et al.*, 2002; (17) CDPR, 2006.*

1.2.1. Imidacloprid mode of action

IMI mode of action is based on the interference of the neurotransmission in the nicotinic cholinergic nervous system. IMI binds to nicotinic acetylcholine receptors (nAChRs) at the neuronal and neuromuscular junctions in insects and vertebrates. The nAChRs are members of the cys-loop ligand-gated ion channel (LGIC) superfamily consisting of five membrane proteins arranged around a central cation permeable pore (Sine and Engel, 2006) (Fig. 5). These ion channels rapidly transduce the actions of the excitatory chemical neurotransmitter acetylcholine (ACh) to membrane depolarisation at the level of synapses. The receptor is normally present in a closed state; however, after ACh binding, the complex channel opens a pore and becomes permeable for cations.

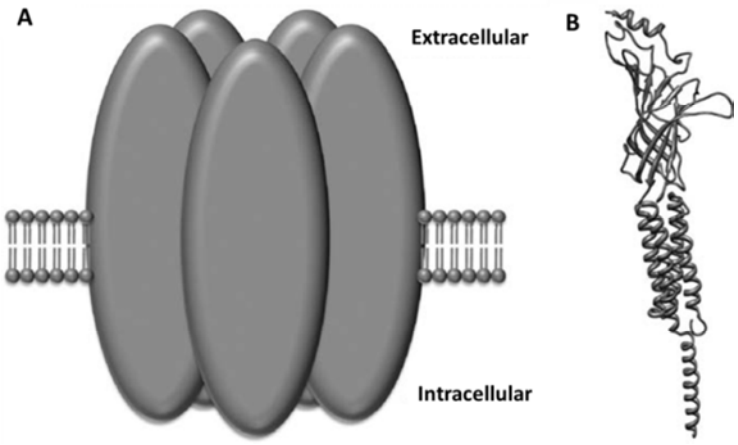


Figure 5: Nicotinic acetylcholine receptors (nAChR) and subunit structure. (A) Schematic representation of nAChR, presenting the pentameric arrangement of subunits around a central cation-permeable pore. (B) Three dimensional structure of an individual nAChR subunit showing the structure of the polypeptide backbone (Millar, 2009).

Similarly to the naturally occurring signal transmitting ACh, IMI stimulates certain nerve cells by acting on a receptor protein. IMI fits to the receptor that normally receives the molecule of ACh and irreversibly blocks postsynaptic nAChRs (Tennekes, 2010) (Fig. 6). In contrast to ACh, which is quickly degraded by enzyme acethylcholinesterase (AChE), IMI is inactivated either very slowly or not at all

(Cox, 2001). Prolonged activation of the nAChR by IMI causes desensitisation and blocking of the receptor and leads to paralysis and death.

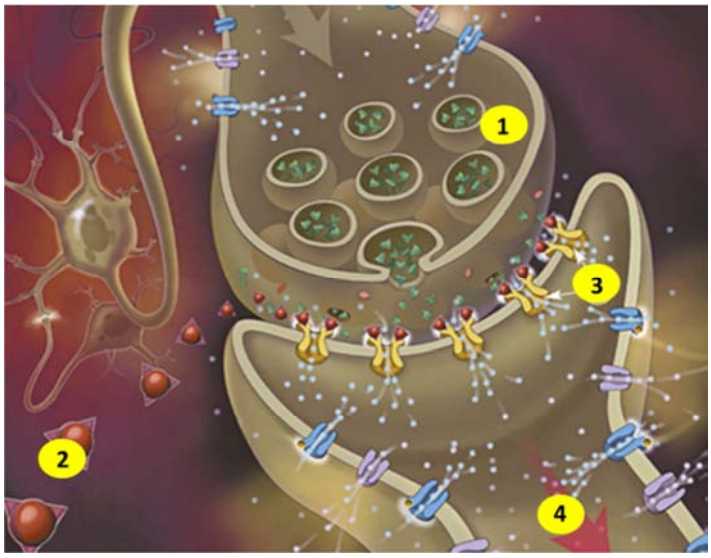


Figure 6: Site and mode of action of imidacloprid on insect neuronal cells. (1) Vesicles of acethylcholine, (2) imidacloprid, (3) nicotinic receptors blocked open and (4) constant neuromuscolar stimulation. (Bayer HealthCare AG®).

To understand the activity of IMI on insect and mammalian nAChRs some structural properties are important. The IMI molecule consists of a 6-chloro-3-pyridyl group and a 2-nitroimino-imidazolidine group, bridged by a methylene group (Fig. 3). Toxicity, binding and receptor activation are affected by all three of these components and also by the overall hydrophobicity of the molecules.

Insect nAChRs - high affinity IMI binding sites in insect nAChRs were detected in a broad range of insects, including green peach aphid, glassy-winged sharpshooter, whitefly, cockroach, migratory locust and fruit fly (Zhang *et al*., 2000; Tomizawa and Casida, 2003). The 2-nitroimino-imidazolidine group plays a key role in the selectivity of insect nAChRs. Neonicotinoids are not protonated and instead of an easily protonated nitrogen they have an electronegative nitro or cyano pharmacophore. This electronegative pharmacophore is proposed to associate with a cationic subsite in the insect nAChRs (Tomizawa and Casida, 2005). In general, the potency of IMI for insect brain nAChRs is considerably higher than for mammalian channels. For example, the binding affinity of IMI to nAChRs of *Drosophila* sp. is

over 550-fold greater than the affinity to the mammalian receptors (Tomizawa, 2001).

Mammalian nAChRs - structural studies suggested that the electron deficient nitrogen atom of the imidazolidine group in IMI molecule corresponds to the protonated form of nicotine and interacts with the mammalian nicotinic receptors (Matsuda *et al.*, 2000). The binding affinity and agonist potency of IMI have been reported for several vertebrate species. IMI showed a partial agonist activity with the recombinant chicken α4β2 and α7 receptors (Matsuda *et al.*, 1998; 2000). IMI presented also agonist action on nAChRs in BC3H1 muscle cells and mouse N1E-115 neuroblastoma (Zwart *et al.*, 1994).

It is important to describe several additional effects that occur at the receptor and cellular level after exposure to IMI. It was demonstrated that IMI (after 3 days of exposure) up-regulates the α4β2 nAChR subtype in mouse M10 cell line (Tomizawa and Casida, 2000). This up-regulation is often associated with the receptor desensitisation (Pauly *et al.*, 1996). At the cellular level, the immediate effect of nAChRs activation is an increase in the level of intracellular calcium and consequent membrane depolarisation which then activates a complex downstream signalling pathway (Berg and Conroy, 2002). One of the key components involved in the nAChR downstream signalling pathway is the extracellular signal-regulated kinase (ERK), also known as mitogen-activated protein kinase (MAPK). In the study of Tomizawa and Casida (2003) IMI activated the ERK cascade in mouse neuroblastoma N1E-115 cells after 30 min incubation. The IMI stimulation of α4β2 receptor was joined with the phosphorylation of ERK in a Ca^{2+} and protein kinase C (PKC)-dependent manner (Tomizawa and Casida, 2003). ERK pathway is a necessary intermediate in the signalling from the nAChR to expression of specific genes (Chang and Berg, 2001). Several human neuropathologies have been linked to genetic alterations of nAChRs genes (Lindstrom, 2002). These receptors are also involved at different levels in several neurodegenerative diseases such as Parkinson and Alzheimer's. There is a growing number of facts (even if studies are not entirely consistent) which correlates long-term low-dose pesticide exposure along with the specific agricultural working conditions to a variety of disease conditions including cancers, reproductive health problems and a range of neurological disorders (Parrón *et al.*, 2011).

1.2.2. Environmental fate and behaviour of imidacloprid

After the application of pesticides used for pest control in agriculture a lot of different transportation and transformation processes may occur. The fate of a pesticide comprises its behaviour in all three major compartment of the environment: air, soil and water. In general, the principal routes of dissipation for IMI in the environment are aqueous photolysis, microbial degradation and uptake by plants.

Air: Some formulations of IMI are applied as sprays, allowing for possible off-site movements through drift. IMI's low vapour pressure (2×10^{-7} Pa at 20º C) and low soil adsorption coefficient indicates that its volatilisation from soil and leaf surfaces may not be the main route of dissipation. Combination of these characteristics with low Henry's law constant of 6.5×10^{-11} atm m^3 mol^{-1} makes it unlikely that IMI will be present in the air in measurable amounts after application by any method. For example, an air monitoring study of IMI residues has been performed by the California Department of Pesticide Regulation (CDPR) in cropped field treated with IMI for control of glassy-winged sharpshooter (*Homalodisca coagulata*). Air samples collected immediately after spray applications in this area did not detect any residues of IMI (Walters *et al*., 2001; Segawa *et al*., 2004).

Soil: The relevant water solubility and low K_{oc} for IMI indicate a low predisposition for adsorption to soil particles (Table 1). Field studies have produced a wide range of half-life values (DT_{50}). DT_{50} is the time required for 50% of the field-applied pesticide to dissipate and in case of IMI can range anywhere from approximately 80 days to 2 years (Table 1). Some authors consider IMI as relatively immobile in soil and do not expect its significant leaching behaviour (Krohn and Hellpointner, 2002), while some studies indicate the opposite (Gupta *et al*., 2002). IMI persistence in soil depends on a wide range of factors such as: soil type, pH, and presence or absence of ground cover. Very important factors, which also influence the mobility, are the chemical nature of active substances and the formulation characteristics (Gupta *et al*., 2002). Furthermore, degradation of IMI in soil is decreased if organic materials used to improve soil quality (organic fertilisers) are added (Rouchaud *et al*., 1996).

Water: Based on solubility of IMI and its persistence, IMI is considered to have high leaching potential. The solubility of IMI in water is relatively high, and its octanol-water partitioning coefficient is quite low (Table 1). Generally, IMI is persistent in water and not easily biodegradable (Tišler *et al*., 2009). The influences of pH and applied formulation on the persistence of IMI in water have also been studied, and it was found that a higher pH, meaning alkaline conditions, increases half-life time and consequently its persistence. At environmentally relevant pH values, hydrolysis of this insecticide is greater than 30 days, but it can be rapidly degraded through photolysis with a half-life of 1-3 hours (Tišler *et al*., 2009).The formulation of the pesticide also presents a significant effect on IMI persistence. For example, the powder formulation Gaucho 70WS was more persistent in water than the liquid formulation Confidor 200SL (Sarkar *et al*., 1999).

1.3. Transformation products of imidacloprid

Pesticides can be transformed in the environment, crops or organisms into a large number of different products, called **transformation products (TPs)**. It is widely known that TPs may have different properties which allow them to occur in areas which usually cannot be reached by a pesticide itself. Due to their higher polarity and mobility in the soil-water environment, TPs may reach surface- and ground-water more easily than the parent compound (Hernández *et al*., 2008). These products can be more toxic (Bavcon Kralj *et al*., 2007) and more persistent than the parent compound (Kolpin *et al*., 2009).

IMI's chemistry is founded on nitromethylene derivatives, the activity or stability of which is modified mainly by three processes: (1) change of the heterocyclic ring to an imidazolidine ring; (2) modification of the nitromethylene moiety to nitroimino; (3) and introduction of a pyridyl moiety (Iwaya and Kagabu, 1998). All transformation processes are related to these three possible pathways.

The TPs of IMI in soil are typically IMI urea, 6-chloronicotinic acid (6CNA) and 6-hydroxynicotinic acid (Scholz and Spiteller, 1992; Rouchaud *et al*., 1996) (Fig. 7). It is important to notice that IMI degradates express mainly higher water solubility and consequently higher leaching or contamination potential of aquatic compartments. 6CNA is one of the final transformation products of IMI and due to its high water solubility (2 g L^{-1}) it may leach from soil into the aquatic environment. IMI's major photolysis breakdown product in water are 6-chloronicotinic aldehyde, 6-chloro-*N*-methylnicotinacidamide, 6-chloro-3-pyrydylmethylethylendiamine, IMI-urea, 6-hydroxynicotinic acid, and a minor breakdown product is IMI-guanidine (Bacey, 2001). The major TPs resulting from incubation of aqueous samples under the environmentally relevant non-sterile conditions and light exposure were desnitro-IMI, IMI-urea and 6CNA (Mulye, 1995) (Table 2). Under dark, non-sterile, anaerobic conditions, desnitro-IMI was the major TP of IMI and has been found to be more persistent than its parent compound (Mulye, 1995). IMI taken up by the crops is metabolised to several major products including desnitro-IMI, 5-hydroxy-IMI and 6CNA (Table 2).

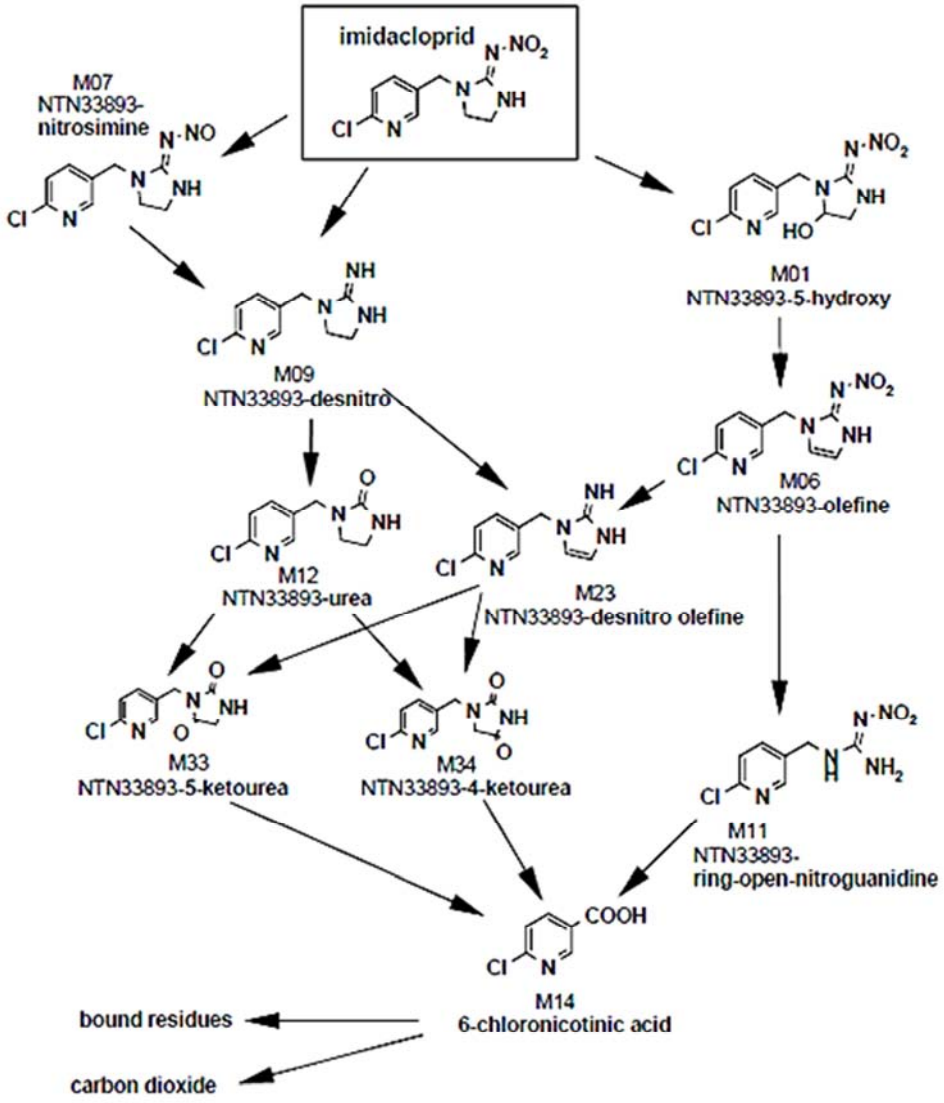
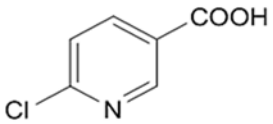
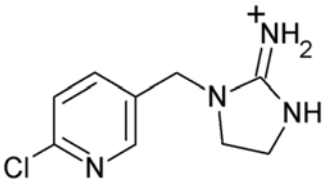
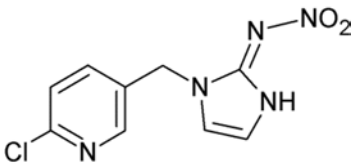
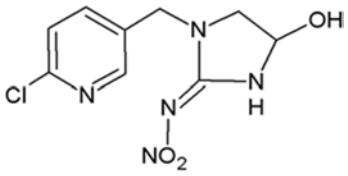


Figure 7: Probable degradation pathway in soil (main TPs) (IMIDACLOPRID (206) by U. Banasiak).

The TPs produced through *in vivo* metabolism of neonicotinoid compounds in animals (i.e. mouse and rat model organisms) are numerous and well known metabolites. The metabolism of IMI is complex and has been studied in goats, rodents and laying hens (Tomizawa and Casida, 2005). Two major routes are present for IMI metabolism in the rat as shown in Fig. 8 (Thyssen and Machmer, 1999). In the first route, IMI undergoes oxidative cleavage to imidazolidine and hydrolysis to 6CNA. The second route involves hydroxylation in the imidazolidine ring (formation of 4- and 5-hydroxy-IMI), followed by elimination of water and formation of olefin-IMI (JMPR, 2001; CDPR, 2006).

Table 2: Chemical structure, IUPAC name and selected properties of four major IMI's TPs: 6CNA, desnitro-IMI, olefin-IMI and 5-hydroxy-IMI.

IMI's TP	*CAS number and chemical structure*	*IUPAC name*	*Molecular weight (g mol^{-1})*	*Solubility (g L^{-1})*
6CNA	5326-23-8 	6-chloropyridine-3-carboxylic acid	157.6	2
desnitro-IMI	115970-17-7 	1-(6-chloropyridin-3-ylmethyl) imidazolidin-2-ylideneamine hydrochloride	247.13	180-230
olefin-IMI	1115248-04-8 	1-(6-chloro-3-pyridylmethyl)-*N*-nitro-1,3-dihydro-*2H*-imidazol-2-ylideneamine	253.65	> 0.51
5-hydroxy-IMI	1115248-02-6 	(E)-3-(6-chloro-3-pyridylmethyl)-2-(nitroimino) imidazolidin-4-ol	271.66	> 0.51

Different studies performed with IMI and its TPs indicated that the desnitro- or guanidine-TPs may be activators of toxicity in mammals and detoxification products in insects, while the opposite is true for the olefin- and nitrosoimine-TPs (Schulz-Jander and Casida 2002; Schulz-Jander *et al*., 2002). One of these TPs, desnitro-IMI (Table 2), is of particular interest, because it presents preference for mammalian versus insect nAChRs and could be related with possible toxic effect also in humans (Tomizawa and Casida, 1999; Tomizawa and Casida, 2000). Important enzymes in IMI biotransformation are cytochrome 450 (liver microsomal CYP450) and cytosolic aldehyde oxidase (AOX) (Honda *et al*., 2006). CYP3A4

oxidises the imidazolidine moiety and reduces the nitroguanidine substituent (Schulz-Jander and Casida 2002; Schulz-Jander *et al*,. 2002) (Fig. 7), while AOX reduces the nitroguanidine moiety of IMI (Schulz-Jander *et al*., 2002; Dick *et al*., 2005). Metabolism of the neonicotinoid can decrease or increase its toxicity/potency, based on the compound and specificity of the nAChR.

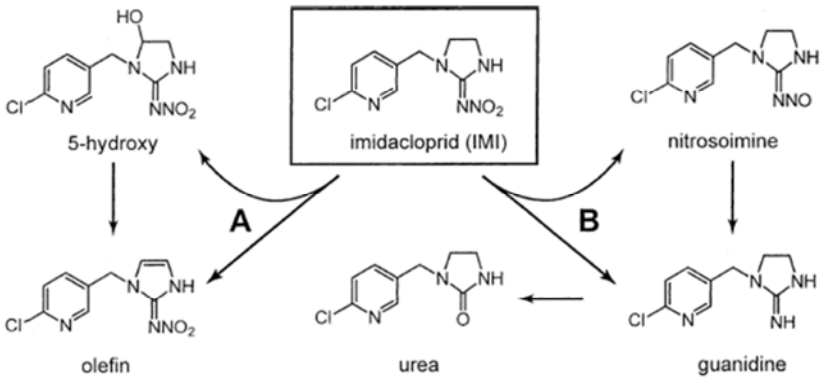


Figure 8: IMI metabolism by human CYP450 (A) hydroxylation or desaturation of imidazolidine moiety with formation of hydroxyl- and olefin-IMI; (B) cleavage and reduction of nitroimine substituent to form urea, nitrosoimine and guanidine metabolites. (Schulz-Jander and Casida, 2002).

The safe and effective use of pesticides requires a clear understanding of the parent compound, but also of its TPs. All legislations related to the placement of pesticides on market focus only on parent compounds rather than marketed formulations or TPs. No particular control or monitoring over the presence of IMI TPs is performed and there is a great necessity to implement this part of legislation by establishing the MRLs levels for several TPs present in the environment. Although IMI is continuously studied, fewer investigations have been conducted on its TPs. There is a single study relative to the toxicity of 6CNA on aquatic invertebrates performed on midge *Chironomus tentans* with LC_{50} (96 h) higher than 1 mg L^{-1} (Bowers and Lam, 1988). There is a great need to improve our knowledge relative to IMI's TPs by understanding their possible toxic effects to non-target organisms. In this study in addition to the selected parent compound **IMI** and its **commercial formulation Confidor 200SL**, four major TPs of IMI: **6CNA**, **olefin-IMI**, **desnitro-IMI** and **5-hydroxy-IMI** were also included (Fig. 4).

1.4. Pesticide effects on different levels of biological organisation

Pesticides released into the environment may have several adverse ecological effects ranging from long- to short-term effects in the ecosystem. Pesticides hold a distinctive position among environmental contaminants due to their acute and chronic toxicity. After the application of the pesticides (with spraying through sprinkling or by wetting the seeds or crops); the pesticides can influence different soil or aquatic organisms (Fig. 9). The indirect influence of pesticides to this group of organisms called **non-target organisms** should not be neglected. Compounds currently applied in agriculture are biologically very active and their specificity could not be improved to such extent that effects on non-target species can be avoided (Schulz *et al*., 2002).

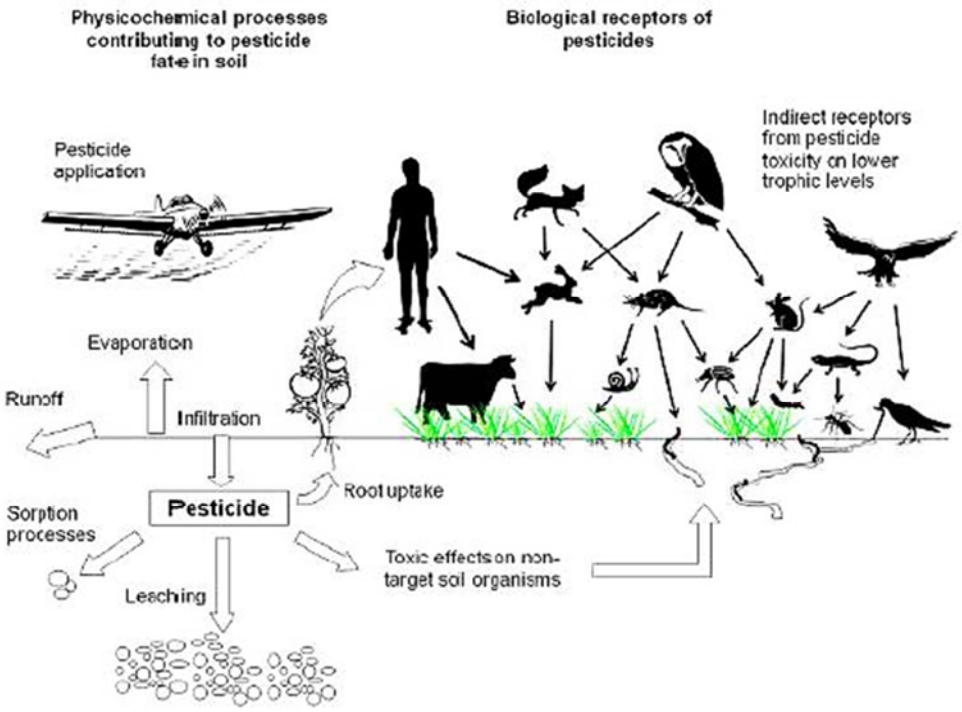


Figure 9: The general scheme of pesticides environmental fate and influence of different organisms in the environment (Sanchez-Hernandez, 2011).

The toxicity of a pesticide is determined by quantifying **biomarkers** (or **conventional endpoints**) of experimental organisms to a series of increasing chemical's doses in different **toxicological bioassays**. This relationship between administered dose and organism response is graphically represented as the dose-response curve (Fig. 10).

A common measure used to define toxicity at a certain dose is the $\mathbf{LD_{50}}$ ($\mathbf{LC_{50}}$) – median lethal dose or concentration at which more than 50 % of organisms die. The $\mathbf{EC_x}$ is the effective concentration of test sample at which x % (e.g. 10, 20 or 50 %) of its maximum effect is reached.

The specific point on the dose-response curve where the more susceptible animals are first affected by a pesticide dose is termed the **threshold level**. The threshold level is the beginning of the linear response region of the curve and is the separation between the "No Observed (Adverse) Effect Level/Concentration" (**NO(A)EL/NO(A)EC**) and the "Lowest Observed (Adverse) Effect Level/Concentration" (**LO(A)EL/LO(A)EC**). The NO(A)EC value is the highest tested concentration that does not yet cause a statistically significant effect compared to the control, while the LO(A)EC value is the lowest tested concentration that elicits a statistically significant effect compared to the control.

In addition, the effects of a pesticide vary not only with the concentration/dose but also with the duration of exposure that can be:

- acute (short-term exposure with high concentrations)
- sub-chronic (intermediate-term exposure)
- chronic (long-term exposure with low concentrations)

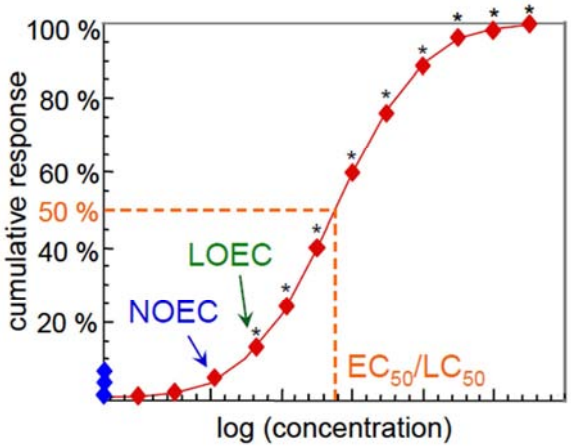


Figure 10: General scheme of a dose response curve with the toxicity parameter NOEC, LOEC and EC_{50}. ◊ Control, ◊ treatment, * significant difference compared to control (Kienle et al., 2011).

The use of NOEC and LOEC data in regulatory aspects of ecotoxicology has been severely criticised since a series of published data in the 1990s (Hoekstra and Van Ewijk, 1992; Noppert *et al*., 1994; Chapman *et al*., 1996; OECD, 1998). Although an OECD (1998) workshop recommended that NOECs should be discarded out of international standards, guidance and toxicity testing, NOEC and LOEC estimations still persist in more recent OECD statistical publications (2006) because "*…the NOEC is still required in many regulatory standards from many countries and in some cases where a detailed determination of an ECx is not relevant and the alteration of the study design is too costly to fullfil the requirements for regression models*". In general, despite these criticisms, NOECs and LOECs are continuously generated and reported regularly in the literature since no standardised alternative is completely accepted.

Environmental risk assessment (ERA) of environmental chemicals (e.g. pesticides) is predominantly based on the results of laboratory toxicological bioassaya and studies as previously explained. This assessment is typically based on a comparison of effect estimations and exposure concentrations. ERA is completed in four steps: hazard identification, dose-response assessment, exposure assessment, and risk characterisation (EC, 2003). Besides ERA, there is another interesting term in this field known as: Integrated Pest Management (IPM) which was formalised by

the US Academy of Sciences in 1969. IPM is largely a combination of the integrated control, which combines biological and chemical control of pests with pest management. In addition, the European Commission is promoting low pesticide-input farming in Member States with the application of IPM. IPM relies on minimising pesticide use through the implementation of alternative methods to control pests, diseases and weeds. Community-wide standards for IPM are being developed and will be mandatory across the EU from 2014 (Hillocks, 2012).

In general, toxicity testing is conducted to determine the potential human health and environmental hazards of chemicals (e. g. pesticides) and their products. Human toxicology, by definition, is concerned with the effects of chemicals on a single species: man. In this case the research and testing are undertaken with one major objective, protection of the health of the individual. Pesticides are considered as a significant source of diverse pollutants that can cause health implications in humans (Hellweg and Geisler, 2003). Contrary to most other chemicals, they are deliberately emitted to the environment to control undesirable organisms, but can also reach non-target life forms such as humans. We are exposed to pesticides in the food, water and air we use and in these cases the duration of exposure, the role of pollutant mixtures, the mode of action should be evaluated and pesticide use in agriculture must be subjected to continuous monitoring.

In contrast to the human toxicology is ecotoxicology, which not only deals with the impact of chemicals on individuals of different species, but also deals with the influence of chemicals on supra-organismal levels. Ecotoxicology has evolved mainly from three different disciplines: toxicology, applied ecology and environmental chemistry. It as an interdisciplinary environmental science that deals with the interactions between environmental chemicals and biota, thereby focusing on adverse effects at **different levels of biological organisation** from the molecular, cellular, and organism level, up to populations and ecosystem (Fig. 11). Ecosystems include a large numbers of species and pesticides effect may be identified at different biological levels, i.e. individuals of a species, populations of species and groups of species (communities) with trophic links. Pesticide effect can be simple/direct or indirect due to complex food web interactions at higher levels of biological integration (Preston and Snell, 2001; Preston, 2002). Direct effects may depend on

the mode of action of the compound (Barata and Baird, 2000), the concentrations (Naddy *et al*., 2000), and duration of the exposure (Reynaldi and Liess, 2005). These straight-forward effects of pesticide exposure on susceptible species include lowering of survival rate (Barry *et al*., 1995), or effects on different sub-lethal endpoints such as food consumption, growth (Hooper *et al*., 2005), and brood production (Hosmer *et al*., 1998). Pesticide exposure affects swimming behaviour, filtration rates as well as predator-prey relationships (Dodson *et al*., 1995). After pesticide application was noted also reduction in diversity, density, and biomass of many invertebrate species in aquatic communities (Berenzen *et al*., 2005; Schafer *et al*., 2007).

Currently there are present two main approaches that reflect the trends in eco-toxicological researches. One way is to work in real ecosystems which are represented by simplified but still realistic model systems like mesocosms. In these models are simulated and investigated complex interactions and feedback mechanisms which occur in ecosystems. An alternative approach is to develop a mode of action based effect evaluation (Escher *et al*., 1997) by investigating responsible molecular mechanisms of toxic action. These modes of action can be solved using **whole-organism (*in vivo*)** or ***in vitro*** model systems in order to develop and measure specific **biomarkers** that can be applied as early warning signals. In eco-toxicological research, cellular and biochemical effect studies including mechanisms of toxic action are equally important as studies in cell culture and whole organism models because the primary interaction between chemicals and biota occurs at the surface of/or in cells. In this way, cellular toxicology provides an essential concept in understanding eco-toxicological processes, as it plays a key role in identifying toxicological effects at higher biological levels. Taken together, these two described approaches integrate each other and will finally improve the understanding of the effects of pollutants (e.g. IMI) on living systems (biota and humans).

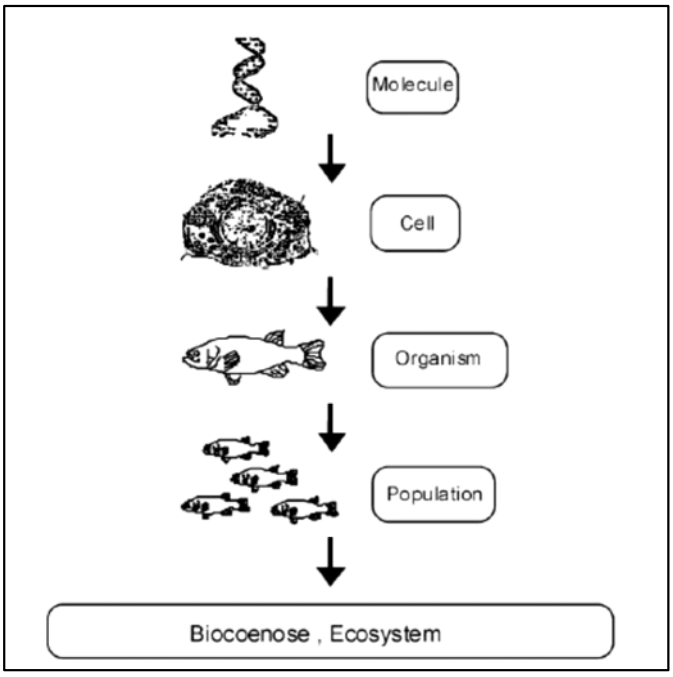


Figure 11: Ecotoxicology, an environmental science focusing on different levels of biological organisation (Fent, 2001).

1.4.1. Imidacloprid - toxicity studies on non-target aquatic organisms

IMI can contaminate surface- and ground-water by runoff or leach from agricultural areas and lead to pulse or localised pesticide contaminations (Fossen, 2006; Gupta *et al*., 2002). Detected aquatic concentrations indicate measured levels of IMI going from 14 μg L^{-1} up to 0.3 mg L^{-1} for surface waters, while the estimated concentrations for accidental spills reach high values going from 1.8 mg L^{-1} up to 7.3 mg L^{-1} (Table 1).

IMI based on its mode of action is in specific toxic to insects and aquatic insects; more than to other aquatic invertebrates (Overmyer *et al*., 2005), crustaceans and fish (Tišler *et al*., 2009). Freshwater fish do not appear to be particularly sensitive to IMI, with toxic effects occurring at relatively high concentrations over 80,000 μg L^{-1} (CCME, 2007). In specific, only the early life stages of fish exhibit higher sensitivity (Cox, 2001). In case of freshwater plants there is evidence that IMI may be harmful to plants and algae at higher concentrations (Table 3). The acute toxicity of IMI was tested on different species of **green algae** (Heimbach 1989; Gagliano and Bowers 1991). These studies gave a NOAEC and EC_{50} values for biomass and growth higher than 119 mg L^{-1} a. i. for 5 day test with *Selanastrum capricornutum* and 10 mg L^{-1} a.i. for *Scenedesmus subspicatus*. In comparison, a 4 day NOEC of 6.69 mg L^{-1} and 4 day LOAEC of 9.98 mg L^{-1} was determined for the diatom (*Navicula pelliculosa*) after exposure to IMI formulation with 21.6 % of a. i. (Hall, 1996). In general, it appears that algae are at least three orders of magnitude less sensitive to IMI than many insect and ostracod species.

Furthermore, the major part of studies referring to the persistence and toxicity of IMI in the aquatic environment is focused to different **non-target aquatic invertebrates** (Table 4). IMI induces toxic effects in several aquatic invertebrates at different concentrations having a species-specific action and effects. Using survival and growth endpoints a 96 h and 10 day NOEC values have been determined at 1.24 μg L^{-1} a. i. and 0.67 μg L^{-1} a. i., respectively for *Chironomus tentans* (Gagliano, 1991). Effects at such low concentrations of IMI were reported also by Stoughton (2008) for the same species, with a LC_{50} for 96 h of 5.75 μg L^{-1} a. i. The sensitivity of crustaceans to IMI appears to vary. A recent study noted that ostracod species seem to be more sensitive than cladocerans e.g. water flea *Daphnia magna* (Sánchez-

Bayo and Goka, 2006). Amphipods appear to have intermediate sensitivity in comparison with other crustaceans. Stoughton (2008) reported in case of the amphipod *Hyallela azteca* a LC_{50} for 96 h of 65.4 μg L^{-1} of a. i. IMI. For the amphipod *Gammarus pulex*, a 28 day LOEC for mortality endpoint was determinate at 256 μg L^{-1} of a. i. IMI (Hendel, 2001). In a 19 week microcosm study IMI was applied to the surface of tanks containing a variety of phytoplankton, zooplankton, and macro-invertebrates at two week intervals with concentrations ranging from 0 to 0.180 mg L^{-1} a. i. Statistically significant decreases in populations of total macro-invertebrates as well as individual taxa (mayflies, midges, beetles and amphipods) were most frequently observed at IMI's concentrations ranging from 0.02 to 0.180 mg L^{-1} a. i.

All investigations span from single species toxicity tests in laboratory (Jemec *et al*., 2007; Chen *et al*., 2010; Lukančič *et al*., 2010; Azevedo-Pereira *et al*., 2011) to complete indoor/outdoor stream mesocosms studies under constant exposure (Pestana *et al*., 2009) and short-pulse exposure conditions (Stoughton *et al*., 2008; Mohr *et al*., 2012). The authors observed usually modifications in survival, behaviour and population growth rate; while some of them evaluated biochemical alterations at molecular level and compared the toxic effects of pure compound IMI and its formulated version (Jemec *et al*., 2007; Chen *et al*., 2010).

Table 3: Selection of some representative IMI toxicity studies on freshwater algae.

Test organism	*Exposure/measured endpoint*	*Toxicity data*	*References*
Green algae *Scendesmus subspicatus*	• sub-chronic toxicity • concentrations of 0, 0.1, 1, and 10 mg L^{-1} • biomass and growth	72 and 96 h EC_{50}: > 10 mg L^{-1} 72 and 96 h NOAEC: 10 mg L^{-1}	Heimbach, 1989
Green algae *Selanastrum capricornutum*	• chronic toxicity • nominal (measured) concentrations of 0, 15.6 (14.1), 25.9 (24.1), 43.2 (41.1), 72 (69.5), and 120 (119) mg L^{-1} • biomass and growth	EC_{50} (5 d): >119 mg L^{-1} NOAEC (5 d): > 119 mg L^{-1}	Gagliano and Bowers 1991
Green algae *Pseudokirchneriella subcapitata*	• chronic toxicity • growth inhibition	EC_{50} (5 d) and NOEC: < 119 mg L^{-1}	Gagliano and Bowers, 1991
Diatom *Navicula pelliculosa*	• sub-chronic toxicity • concentrations: 0, 0.16, 0.42, 1.05, 2.64, 6.69, and 17.0 mg L^{-1} • growth inhibition	4 d NOAEC: 6.69 mg L^{-1} 4 d LOAEC: 9.88 mg L^{-1} 4 d EC_{50}: 12.37 mg L^{-1}	Hall, 1996
Blue-Green algae *Anabaena flosaquae*	• sub-chronic toxicity • concentrations of 0, 24.9, 40.5, 68.2, 121.3, and 193.3 mg L^{-1}	EC_{50} (4 d): 32.8 (30.4-34.6) mg L^{-1} NOEC (4 d): 24.9 mg L^{-1}	Bowers, 1996; Mulye, 1997

Table 4: Selection of some representative IMI toxicity studies on freshwater invertebrates.

Test organism	*Exposure/measured endpoint*	*Toxicity data*	*References*
ACUTE/SUB-CHRONIC TOXICITY			
Water Flea *Daphnia magna* Mosquito *Aedes aegypti*	• static 48 h acute toxicity test • 3 trials, 4 replicates per concentration, 10 animals each species per replicate • mortality	Water Flea: LC_{50} (48 h): 10.44 mg L^{-1} Mosquito: LC_{50} (48 h): 0.044 mg L^{-1}	Song *et al*., 1997 Song and Brown, 1998
Amphipod crustacean *Hyalella azteca*	• static sub-chronic toxicity test • 2 replicates per concentration, 10 per replicate • mortality, immobilisation, abnormal effects (e.g. lethargy or surfacing)	(a) LC_{50} (96 h): 0.526 mg L^{-1} EC_{50} (96 h) for immobilisation : 0.055 mg L^{-1} NOAEC (96 h) for immobilisation and abnormal effects: 0.00035 mg L^{-1} (b) LC_{50} (96 h) for juveniles: 65.43 µg L^{-1} (IMI) LC_{50} (96 h) for juveniles: 17.44µg L^{-1} (Admire) NOEC (96 h) similar for the two products; 54.24 µg L^{-1} (IMI); 48.75 µg L^{-1} (Admire).	(a) England and Bucksath, 1991 (b) Stoughton *et al*., 2008
Midge *Chironomus tentans*	• static sub-chronic toxicity test • 2 replicates per concentration, 10 chironomids per replicate • mortality	LC_{50} (96 h): 0.0105 mg L^{-1} NOAEC (96 h) for survival: 0.00124 mg L^{-1}	Gagliano, 1991
Water louse *Asellus aquaticus* L. Stream scud *Gammarus fossarum* Koch.	• static acute 48 h toxicity test • mortality • electron transport system (ETS) activity and respiration level	Water louse (a.i. in Confidor 200SL): LC_{50} (48 h): 8.5 mg L^{-1} EC_{50} (24 h): 0.8 mg L^{-1} Stream scud (a.i. in Confidor 200SL; 20 % of a. i. IMI). LC_{50} (48 h): 0.8 mg L^{-1} EC_{50} (24 h): 0.07 mg L^{-1}	Lukančič *et al*., 2010

Table 4: Selection of some representative IMI toxicity studies on freshwater invertebrates (continued).

Test organism	*Exposure/measured endpoint*	*Toxicity data*	*References*
ACUTE/SUB-CHRONIC TOXICITY			
Midge *Chironomus tentans*	• constant sub-chronic toxicity test with larvae • mortality	LC_{50} (96 h): 5.75 µg L^{-1} (IMI) LC_{50} (96 h): 5.4 NOEC (96 h): 1.03 µg L^{-1} (IMI) and 5.11 µg L^{-1} (Admire) The difference between the effects of IMI and Admire (commercial formulation) was not significant	Stoughton *et al*., 2008
Microcrustacean *Ceriodaphnia dubia* Richard	• acute toxicity test • survival • population size	LC_{50} (48 h): 2.07 µg L^{-1} (Admire Pro; 42.8% a. i. IMI) 8 days' exposure to a mixture of the nonylphenol polyethoxylate, R-11 and IMI resulted in a population size 3 times smaller than with R-11 alone, and 13 times smaller than with IMI only	Chen *et al*., 2009
Planktonic cladoceran *Chydorus sphaericus*	• acute toxicity • mortality	LC_{50} (24 h): 161.95mg L^{-1} LC_{50} (48 h): 132.67 mg L^{-1} EC_{50} was also determined, and was 2-13 times lower under dark than under normal (16 h light : 8 h dark) laboratory conditions	Sánchez-Bayo and Goka, 2006
CHRONIC TOXICITY			
Water flea *Daphnia magna*	• chronic 21 d toxicity test • 4 replicate per concentration, 6 daphnids per replicate • mortality, immobilisation	(a) EC_{50} (21 d) for immobilisation: >7.3mg L^{-1} LOAEC: 3.6 mg L^{-1} 3.6 and 7.3 mg L^{-1}: Significantly reduced adult daphnid length compared with control 7.3 mg L^{-1}: significantly reduced survival; significantly reduced reproduction compared with control	(a) Young and Blakemore, 1990

Table 4: Selection of some representative IMI toxicity studies on freshwater invertebrates (continued).

Test organism	*Exposure/measured endpoint*	*Toxicity data*	*References*
CHRONIC TOXICITY		(b) NOEC (21 d): 1.25 mg L^{-1} The toxicity of commercial formulation Confidor SL 200 was intensified in comparison to the a.i. (c) LC_{50} (10 d): 9 mg L^{-1}	(b) Tišler *et al*., 2009 (c) Sánchez-Bayo and Goka, 2006
Midge *Chironomus tentans*	• chronic 28 d toxicity test • constant exposure	LC_{50} (28 d): 0.91 µg L^{-1} (a.i. in Admire) NOEC (28 d): 1.14 µg L^{-1} (Admire) NOEC (96 h pulse exposure): 3.47 µg L^{-1} after 10 d	Stoughton *et al*., 2008
Amphipod crustacean *Hyalella azteca*	• chronic 28 d toxicity test • constant exposure	LC_{50} (28 d): 7.08 µg L^{-1} NOEC (28 d constant exposure): 3.44 µg L^{-1} (Admire) NOEC (96 h pulse exposure): 11.93 µg L^{-1} after 10 d	Stoughton *et al*., 2008
MESOCOSMOS phytoplankton, zooplankton, macroinvertebra tes *(Hyalella azteca)*	• 19 week microcosm study • 4 surface applications at 2-week intervals	Amphipods were the most sensitive species, significant decrease in populations of total macroinvertebrates and individual macroinvertebrate taxa. Study recommended 0.006 mg L^{-1} as NOEC for regulatory action.	Morning *et al*., 1992
Macroinvertebrates	• 20 d mesocosmos experiment • three pulses of IMI (duration of 24 h)	IMI pulses reduced invertebrate abundance and community diversity compared to control. Reduction of oxygen consumption in stoneflies.	Pestana *et al*., 2009
Macrozoobenthos community	• 3-week mesocosmos experiment • 3 IMI pulses of 12 h (1 week apart)	Caddisfly *Neureclipsis* sp. reacted after first pulse and most sensitively. Insect larvae (ephemerids and dipteran) negatively affected after repeated pulses. Effects on insects larvae more pronounced in summer, due to increased temperature. Emergence is the most sensitive endpoint.	Mohr *et al*., 2012

1.4.2. Imidacloprid - toxicity studies on non-target mammalian models

Toxicity testing on mammalian experimental models is necessary to completely elucidate possible IMI adverse effects, specific mechanisms of actions and molecular pathways. **Humans** are also a **non-target group** which could be affected by IMI action through common routes of exposure. Diet is the dominant exposure pathway for pesticides (McKone *et al*., 2007; Lu *et al*., 2008). In general, food ingestion, air inhalation and ingestion of drinking water are possible ways of insecticide intake by general public. In addition, agricultural workers are one of the particularly exposed groups to acute (high) and chronic (low) concentration of pesticides after continuous applications, spraying and accidental spillage. Exposure to pesticides has detrimental effects on human health and is considered as the major contributor to most diseases of great public significance.

According to World Health Organisation (WHO) IMI is considered as moderately toxic and its classified by US EPA system as toxicity class II and class III agents because it blocks specific neuron pathway which are more abundant in insect than warm blooded animals. However, several reports have shown high clinical toxicity of IMI in human suicidal attempts (Wu *et al*., 2001; Proenca *et al*., 2005; David *et al*., 2007; Shadnia and Moghaddam, 2008). The oral LD_{50} of IMI is of 450 mg/kg body weight (b.w.) in rats and 131 mg/kg b. w. in mice (Najafi *et al*., 2010). Experimental animals presented signs of toxicity at doses lower than those causing mortality, regardless of the species, formulation or TP administered. In most studies, clinical signs of toxicity, including staggering, sedation, apathy, tremors and convulsions were observed shortly after dosing; these signs were typically resolved in all animals before the end of a study (within 14 days). Temporary decrease in body weight was also a common symptom of IMI-treated animals. In fact, the principal toxicological finding in sub-chronic and chronic oral testing was the reduction in body weight. Monitoring of body weight provides information on the general health status of animals which can be also important explanation of reproductive effects (Aly *et al*., 2009). Moreover, the liver was the principal target organ as demonstrated by the hepatic necrosis or hypertrophy in rats and dogs, elevated activities of serum enzymes, hepatic mixed-function oxidase and alteration of clinical chemistry parameters such as triglycerides, cholesterol and the blood

clotting time (CDPR, 2006). There are some reports showing IMI being negative in a battery of genotoxicity tests such as test for DNA damage and repair capacity, or *in vitro* gene mutation tests and *in vivo* chromosomal aberration tests (JMPR, 2001). On contrary, in some mammalian *in vitro* tests IMI caused sister chromatid exchange and chromosomal aberrations at doses which confirmed to have also cytotoxic effect (JMPR, 2001). Regarding reproductive and developmental toxicity for IMI were reported disproportionally high number of male foetus and lower foetal body weight in rats (CDPR, 2006). The offspring of treated rat mothers exhibited also significant sensory-motor impairments after 30 post-natal days and this effect was associated with increased AChE activity in brain and in plasma (Abu-Donia *et al*., 2008**).** An acute oral neurotoxicity study performed by Sheets (1994) suggested that neurobehavioral and pathological effects are seen only after high dose exposure to IMI, while all noted effects at lower doses were not on the nervous system (SERA, 2005).

Throughout the scientific literature there are present different studies with mammalian models relative to IMI toxicity and induced morphological and pathological alterations. These studies report LD_{50} as well as relative NOEC and LOEC values for different measured endpoints. Only recently the research in this area is also focusing on IMI's mediated toxicity which involves excessive production of reactive oxygen species (ROS), induced oxidative stress and different activated molecular pathways. These current studies include experimental organisms and different mammalian or other cellular models (Table 5).

Table 5: Selection of some representative IMI toxicity studies on mammalian organisms and different neuronal cell models.

Test organism	*Exposure/measured endpoint*	*Toxicity data*	*References*
ORGANISM MODEL			
Central nervous system and liver of rats	• injected intravenously; 2 h exposure at 3/4 of LD_{50} (424-475 mg/kg/b.w.) • antioxidant enzymes • lipid peroxidation (LP) • nitric oxide induction	Induction of nitric oxides in liver. Significant LP. Different responses of CAT, SOD, GPx. Up-regulation of inflammatory cytokines. Results suggested that IMI cause oxidative stress and inflammation in central nervous system and liver.	Duzguner and Erdogan, 2010
Male mice	• oral administration; 24 h exposure to 1/10 of LD_{50} • antioxidant enzymes • lipid peroxidation (LP) • survival rate (LD_{50})	Increase in LP level and the activities of antioxidant enzymes including CAT, SOD, GPx and GST. G6PD activity remained unchanged, while the level of GSH content was decreased. Vitamin C might ameliorate IMI-induced oxidative. Induction of oxidative stress is perhaps the central mechanism by which this pesticide exert its toxic effect.	El-Gendy *et al*., 2010
Female rats (ovaries)	• daily oral administration; 90 days of exposure to 5, 10 and 20 mg/kg/day) • morphology • hormones • antioxidant enzymes	Significant alterations of SOD, CAT, GPx, GSH and LP (at 20 mg/kg b.w.). Significant toxicological impact on ovary of female rats as evident by patho-morphological changes, hormonal imbalance and generating oxidative stress.	Kapoor *et al*., 2011
Male rats	• daily oral administration; 60 days of exposure to 225 and 112 mg/kg b.w. • behaviour • hormone • tissue morphological analyses	Decreased movement, staggering, trembling, diarrhoea and spasms. Increased thickness of *tunica albuginea*, edema in connective tissue, cytoplasmic granulation. Noted decrease in serum testosterone levels.	Najafi *et al*., 2010

Table 5: Selection of some representative IMI toxicity studies on mammalian organisms and different neuronal cell models (continued).

Test organism	*Exposure/measured endpoint*	*Toxicity data*	*References*
ORGANISM MODEL			
Female rats	• daily oral administration; 90 days of exposure to 5, 10 (NOEL) and 20 mg/kg/day) • SOD, CAT, GPx,GSH and LP in liver, brain and kidney	IMI at 5 and 10 mg/kg/day has not produced any changes in SOD, CAT, GPx, GSH and LP. Changes of these parameters were significant at 20 mg/kg/day in liver. Also, alterations of SOD, CAT, GPx in brain and LP in kidney. 10 mg/kg/day considered as NOEL through antioxidant enzymes and LP.	Kapoor *et al*., 2010
Female rats	• daily oral administration; 90 days of exposure to 5, 10 (NOEL) and 20 mg/kg/day) • survival, body weight and biochemical parameters	Decrease in the body weight at 20 mg/kg/day and at necropsy body weights of liver, kidney and adrenal were increased. Elevation of serum enzymes, glucose and decreased the activity of AChE in serum and brain.	Bhardwaj *et al*., 2010
MAMMALIAN NEURONAL CELLS			
Mouse neuroblastoma N1E-115 cell line	• 30 min (0-180 min) incubation (0.0001-1000 μM) • ERK kinase cascade • intracellular calcium (Ca^{+2}) mobilisation	IMI, desnitro-IMI and nicotine induced ERK activation in a dose-dependent manner triggered by primary action at the α4β2 nAChR with an involvement of intracellular Ca^{+2} mobilisations. Intracellular Ca^{+2} activate a sequential pathway from protein kinase C (PKC) to ERK.	Tomizawa and Casida, 2002
Neuronal SH-SY5Y and pulmonary A549 cell lines	• 3 day incubation (0.2-2.7 mM) • growth rate (LOEC, IC50) • cell count, total protein content • heat shock proteins (HSP) • glucose regulated proteins	Neuronal cells more sensitive than pulmonary. For SH-SY5Y: LOEC – 1.2 mM (IMI) and 0.3 mM (Confidor 200SL); IC_{50} – 1.6 mM (IMI) and 0.8 mM (Confidor 200SL). For A549: LOEC – 1.2 mM (IMI) and 0.3 mM (Confidor 200SL); IC_{50} – 1.8 mM (IMI) and 1.3 mM (Confidor 200SL). Commercial formulation (Confidor 200SL) indicates an additional additive toxic effect. Insecticide concentrations higher than IC_{50} were found to induce an under-expression of all cytosolic HSP probably resulting from a general inhibition of protein synthesis	Skandrani *et al*., 2006

Abbreviations: IC_{50} (median inhibition concentration).

Table 5: Selection of some representative IMI toxicity studies on mammalian organisms and different neuronal cell models (continued).

Test organism	*Exposure/measured endpoint*	*Toxicity data*	*References*
MAMMALIAN NEURONAL CELLS			
Mouse fibroblast M10 cells	• 3 day incubation • up-regulation of α4β2 AchRs and mechanisms of up-regulation	IMI up-regulate α4β2 AchRs by five- to eightfold with EC_{50s} of 70,000 and 19,000 nM. On contrary, desnitro-IMI TP gave up-regulation by eightfold and EC_{50}s of 870 and 500 nM, respectively. Determinated IC_{50} for IMI was of 2600 nM and for densitro-IMI of 8.2 nM.	Tomizawa and Casida, 2000a
Primary cultures of cerebellar neurons from neonatal rats	• excitatory Ca^{2+} influx assay • applications of doses between 1, 10 and 100 μM	Significant excitatory Ca^{2+} influxes were evoked at concentrations greater than 1 μM The firing patterns, proportion of excited neurons, and peak excitatory Ca^{2+} influxes showed differences from those induced by nicotine.	Kimura-Koroda, 2012
Cholinergic synapses of the stellate cells of the mouse cochlear nucleus	• determine to what extent IMI affects the nAChRs • exposure doses based on EC_{50} against mammalian nAChRs of 70 μM • whole-cell patch-clamp recording	Puff application of 1 μM had no significant effect on the membrane properties of the neurons, while a concentration of 10 μM caused a significant depolarising shift in the membrane potential. Concentrations >50 μM caused a significant depolarising shift in the membrane potential. Exposure to IMI at concentrations >10 μM for <1 min can change the membrane properties of neurons that have nAChRs and, as a consequence, their function.	Bal *et al*., 2010

Table 5: Selection of some representative IMI toxicity studies on mammalian organisms and different neuronal cell models (continued).

Test organism	*Exposure/measured endpoint*	*Toxicity data*	*References*
OTHER NEURONAL CELL MODELS			
Cultured GFP tagged cholinergic neurons from the third instar larvae of the genetic model organism *Drosophila melanogaster*	• 5 s applications of 100 μM dosed at 5 min intervals • fura-2-based calcium imaging	Dose-dependent increase in intracellular Ca^{+2}. Increase in Ca^{+2} involve also voltage gated calcium channels.	Jepson *et al*., 2006
Neural Kenyon cell of cockroach *Periplaneta americana*	• incubation for 1, 3, 5, 8 and 24 h at 1, 5, 10, 50 and 100 μM • cell viability (Trypan blue) • adenylate kinase activity (AK)	Significant decrease of cell viability after IMI exposure. Increase of AK activity. Higher concentrations of IMI needed for intracellular mechanisms of IMI toxicity.	Benzidane *et al*., 2011
Neurons isolated from the three thoracic ganglia of the cockroach, *Periplaneta americana*	• application of 100 μM for 20 s • agonist actions of on nAchRs • single electrode voltage clamp recording	Strong excitation symptoms followed by prostration and death. Relatively weak partial agonists causing only 20–25 % of the maximum ACh current. Agonist efficacy, but not affinity, was positively correlated with insecticidal activity.	Tan *et al*., 2007
Central nervous system neurons of the stick insect (*Carausius morosus*)	• Brief U-tube application of 10^{-4} M doses for 1 s • patch-clamp experiments • effects on nAchRs	Generally depressive symptoms, characterised by stillness and weakness, while also variably inducing postural changes such as persistent ovipositor opening, leg flexion or extension and abdomen bending that could indicate excitation of certain neural circuits. Selective desensitisation of certain nAChR subtypes can account for the insecticidal actions of IMI.	Oliveira *et al*., 2011

1.4.3. Imidacloprid's transformation products - toxicity studies on non-target organisms

No particular monitoring over the presence of **IMI TPs** is performed. Although IMI is continuously investigated, fewer studies have been conducted on its TPs (Table 6). Major part of studies on IMI's TPs is performed mainly on common honey bee *Apis mellifera* (Nauen *et al*., 2001; Suchail *et al.*, 2001) (Table 6). Bees, including honey bees, bumblebees and solitary bees are the prominent and economically most important group of pollinators worldwide. There is a great concern regarding the decline of *Apis mellifera* across Europe and world. This event has been termed as colony collapse disorder (CCD) (Fig. 12). Although the supposed causes are still currently analysed, the extensive use of chemical pesticides, such as neonicotinoids (e. g. IMI) may have contributed to loss of pollinators. Laboratory studies demonstrate that IMI is acutely toxic to bees with LD_{50} (48 h) values ranging from 3.7 to 230.3 ng bee^{-1} (SERA, 2005). For chronic laboratory tests with IMI was demonstrated a high worker bee loss when honey bees consumed contaminated pollen at 40 μg kg^{-1} and sugar water at 0.1, 1 and 10 μg L^{-1} (Decourtye *et al*., 2001; 2003). Sub-lethal effects have been noted in bumblebees (members of the bee genus *Bombus*) at concentration that could be considered harmless and included altered foraging behaviour, lower reproduction and decreased pollination (Mommaerts *et al*., 2010). Additional sub-lethal effects consist of impairment of olfactory memory, influenced mobility, reduced learning, and a decrease in 'dancing', which leads to reduced foraging activity (Desneux *et al*., 2007). Sub-lethal dose as low as 0.1 ng of IMI per honeybee seemed also sufficient to disturb navigation in honeybees, loss of foragers and consequently loss of food supply to the colony which then continuous weakening (CST, 2003). All these facts seriously imply that concentrations of several parts per billion (ppb) in the diet of social insects can already harm them on colony level. A recently published review of Blacquière *et al*. (2012) summarises the data relative to the neonicotinoids in bees and stresses the fact that TPs of IMI also contribute to the toxicity to beneficial arthropods. At the moment, most studies were conducted on TPs of IMI in bees and showed that olefin-, dihydroxy- and hydroxy-IMI were more toxic based on oral LD_{50} compared to urea-IMI and 6CNA (Nauen *et al*., 2001).

Figure 12: (A) Frames taken from colonies suffering from CCD with few bees and a large numbers of developing larval and pupae bees. (B) Frame taken from a healthy colony full of bees. (Oldroy, 2007).

Based on structural considerations, the following TPs of IMI may be of toxicological significance: 6CNA, 4- and 5-hydroxy IMI, olefin-IMI, desnitro-IMI and the nitrosoimine compound (a contaminant of IMI preparation and its metabolite). All of these compounds contain the 6-chloropyridinyl moiety and are included in the tolerances established for the IMI residues, although they should be considered separately.

The toxicity of the major TPs of IMI (6CNA, 4 - or 5-hydroxy-IMI and olefin-IMI) has not been fully tested in mammals (CDPR, 2006). Several TPs were tested for acute toxicity by oral administration to rats and for their ability to induce point mutations (mostly by Ames test with bacteria *Salmonella typhimurium*). The TPs showed moderate acute toxicity after oral administrations and clinical signs similar to those described after IMI exposure. Overall, they were found to be less toxic than the parent compound, and all tests for genotoxicity gave negative results (SERA, 2005). However, one of the TPs, desnitro-IMI (Table 2), is important, because its mode of action prefers mammalian *versus* insect nAChRs (Tomizawa and Casida, 2000), as already mentioned in section 1.3 of this manuscript. From a toxicological point of view, the formation of the TP desnitro-IMI is of particular interest, as this product presents a clearly higher toxicity to mammals than IMI and is produced as the major degradation product or metabolite in environment and human liver microsomes, respectively (CDPR, 2006).

The predominant TPs associated with toxicity in insects are olefin-, dihydroxy- and hydroxy-IMI. There is present a general IMI toxicity pattern of exposure which involves an immediate onset of neurotoxicity, followed by a delayed mortality, usually 4 h after exposure. It seems based on the data that unmodified IMI may be responsible for the primary neurotoxic effect, while olefin-, hydroxy- and dihydroxy-IMI which appear at approximately 4 h after exposure may be responsible for mortality (SERA, 2005).

Studies in invertebrates showed that the olefin- and hydroxy- compounds had similar acute toxicity as the parent compound, while 6CNA did not act as a nicotinic agonist (Nauen, *et al*., 2001). None of the assessed IMI TPs (urea-IMI, desnitro-IMI and 6CNA) were as acutely toxic as a.i. IMI alone in tests with the midge (*C. tentas*) or amphipod (*H. azteca*) (Bowers 1996; Bowers and Lam 1998; Rooney and Bowers 1996; Dobbs and Frank 1996).

In general, there are a limited number of studies relative to the toxicity of IMI TPs to aquatic invertebrates (Table 6) which warrants expanding our knowledge in this field. To this end, it is necessary to investigate their effects on freshwater biota, especially on non-target aquatic organisms. In particular, these investigations should be performed as a part of the analysis of pesticide impact on vulnerable aquatic ecosystems.

Table 6: Selection of some representative IMI's TPs toxicity studies on non-target organisms.

Test organism/tested TP	*Exposure/measured endpoint*	*Toxicity data*	*References*
MAMMALIAN ORGANISMS			
Rat (olefin-IMI)	• survival • TP purity of 98 %	Acute toxicity LD_{50} for male of 3500 and for female rats of 1100 mg/kg b.w.	JMPR, 2001
Rat (nitroso TP)	• survival • TP purity of 98.1 %	Acute toxicity LD_{50} for male of 1980 and for female rats of 3560 mg/kg b.w.	JMPR, 2001
Wistar rat (nitroso TP)	• 15 males and 15 females • 0, 100, 300 and 1000 ppm for 12 week (~ 13, 35 and 110 mg/kg b.w.)	Water intake was decreased in the groups at 1000 ppm. At 300 ppm, higher lymphocyte counts and lower numbers of polymorpho-nuclear cells were observed. The NOAEL was 100 ppm (part per million), equal to 13 mg/kg b.w. per day	Krötlinger, 1992
Mouse (nitroso TP)	• Oral application (100 mg/kg b.w.) • Animals sacrificed after 24, 48 and 72 h post-dosing • Micronucleus test • TP purity 98.9 %	The 48 h sacrifice group showed a statistically significant increase over controls in micronucleated polychromatics. There may be a weak effect of the tested TP on micronucleus formation under these conditions.	Herbold, 1989
Rat (nitroso TP)	• 3 or 4 males/dose, 2 or 3 females/dose, 7 weeks old • Oral administration at doses of 300, 1000, 1400, 1800 and 2500 mg/kg b.w. (males); and 1400 and 2500 mg/kg b.w. (females) • Drawback: no control used	LD_{50} > 2500 mg/kg. No mortality. Non-specified poisoning symptoms reported at all doses tested. Authors report "the poisoning symptoms were rather different from those seen in the study on a.i. IMI alone.	Nakazato, 1991
BENEFICIAL INVERTEBRATES			
Honey bee (*Apis mellifera*) (4-,5-dihydroxy-, desnitro-,urea-IMI and 6CNA)	• 3 cages of 20 bees, 3 replicates • acute oral toxicity	The LD_{50} values for the TPs were each > 1000 ng/bee (> 10,000 µg/kg).	Suchail *et al*., 2001

Table 6: Selection of some representative IMI's TPs toxicity studies on non-target organisms. (continued)

Test organism/tested TP	*Exposure/measured endpoint*	*Toxicity data*	*References*
BENEFICIAL INVERTEBRATES			
Honey bee (*Apis mellifera*) head membrane preparations (6CNA, urea-IMI, 4-hydroxy-, 4,5-dihydroxy-IMI)	• binding studies with IMI TPs to determine displacement of ^{3}H IMI	No effective displacing of IMI from its binding site by 6CNA and urea-IMI even at high concentrations (0.1 mM). The affinity of the other TPs for the IMI binding site decreased in the following order: olefin-IMI > 4-hydroxy-IMI >> 4,5-dihydroxy-IMI. 6CNA and urea-IMI are not biologically active via the IMI receptor in the honey bee. Results were confirmed by electrophysiological studies.	Nauen *et al*., 2001
Honey bee *(Apis mellifera)* (5-hydroxy-IMI)	• 20 late summer worker bees of unknown age per treatment, 3 replicates • oral administration	48 h LD_{50}: 153.5 ng/bee (125.9 - 196.9)	Decourtye *et al*., 2003
Honey Bee (*Apis mellifera*) (5-hydroxy-IMI)	• newly emerged worker bees, 60-163 bees per treatment • nominal concentrations of 0, 7.5, 15, 30, 60, 120, and 140 µg/kg sucrose solution • chronic mortality(11 d exposure)	NOAEC (mortality): 120 µg/kg LOAEC (mortality); 240 µg/kg	Decourtye *et al*., 2003
Honey Bee (*Apis mellifera*) (5-hydroxy-IMI, desnitro-IMI, olefin-IMI and urea-IMI)	• 3 cages of 30 bees per experiment, 3 replicates • 10-day chronic mortality study • tested concentrations of 0, 0.1, 1, and 10 ug L^{-1} (0.010, 0.1 and 1 ng /bee/day)	All TPs caused mortality within 72 h after the onset of intoxication (trembling, tumbling, coordination problems). 50% mortality was reached by day 8 for all tested TPs. Except 0.1 ug L^{-1} 5-hydroxy-IMI (reached 40% mortality by end of study). All TPs had similar timing of mortality. 5-hydroxy-IMI showed evidence of dose-response.	Suchail *et al*., 2001

Table 6: Selection of some representative IMI's TPs toxicity studies on non-target organisms. (continued)

Test organism/tested TP	*Exposure/measured endpoint*	*Toxicity data*	*References*
AQUATIC INVERTEBRATES			
Amphipod crustacean (*Hyalella azteca)* (desnitro-IMI)	• 14-21 d old, 10 organisms per concentration, 2 replicates • 96 h static acute toxicity of • measured concentrations of 0, 5.6, 11.0, 22.1, 43.8 and 86.8 mg L^{-1}	96 h LC_{50}: 51.8 mg a.i L^{-1}. 96 h EC_{50} (immobilisation): 29.0 mg a.i. L^{-1}. 96 h NOAEC (mortality): 22.1 mg a.i. L^{-1}	Rooney and Bowers, 1996
Amphipod crustacean (*Hyalella azteca*) (urea-IMI)	• 7-21 d old, 10 organisms per concentration, 2 replicates • 96 h static acute toxicity of • nominal (measured) concentrations of 0, 6.2 (5.81), 12.5 (11.80), 25 (23.46), 50 (46.80), and 100 (94.83) mg a.i. L^{-1}.	96 h LC_{50}: > 94.83 mg a.i L^{-1}, 96 h EC_{50} (immobilisation): > 94.83 mg a.i L^{-1} 96 h NOAEC: 94.83 mg a.i. L^{-1}	Dobbs and Frank, 1996a
Midge (*Chironomus tentans*) (desnitro-IMI)	• 2 replicates, 10 chironomids per replicate • 96-hour static acute toxicity • nominal (measured) concentrations of 0, 0.1, (0.12), 1.0 (0.87), 10.0 (8.19) and 100 (82.8) mg a.i L^{-1}	96 h LC_{50}: >82.8 mg a.i L^{-1} 96 h EC_{50} (sub-lethal effects): 17.0 mg a.i.L^{-1} 96 h NOAEC (mortality and sub-lethal effects): 8.19 mg a.i. L^{-1}. Sub-lethal effects included spotted coloration and erratic behaviour.	Bowers, 1996
Midge *(Chironomus tentans)* (urea-IMI, 6CNA)	• 16 d old, 10 chironomids per concentration, 2 replicates • 96 h static acute toxicity • urea-IMI and 6NCA at concentrations of 0, 0.1 (0.10), 1 (1.0), 10 (10.04) and 100 (99.80) mg a.i. L^{-1}	Urea-IMI: 96 h LC_{50}: > 99.80 mg a.i. L^{-1}, 96 h EC_{50} (sub-lethal effects): >99.80 mg a.i L^{-1}, 96 h NOAEC: 99.80 mg a.i. L^{-1} 6CNA: 96 h LC_{50}: > 1 mg a.i. L^{-1} NOAEC=1 mg a.i. L^{-1}	Dobbs and Frank, 1996b Bower and Lam, 1988

Table 6. Selection of some representative IMI's TPs toxicity studies on non-target organisms. (continued)

Test organism/tested TP	*Exposure/measured endpoint*	*Toxicity data*	*References*
CELL MODELS			
Hamster V79 Cells (nitroso TP)	• doses: -/+ S9: 0.1, 0.3, and 1 mg mL^{-1} • cultures harvested at 7 (high dose only), 18 and 28 h (high dose only) after start of the exposure • chromosomal aberration test	Cytotoxicity of nitroso TP at the mid and high dose indicated by a decline in mitotic index and at the high dose by a decline in plating efficiency (-S9 only). No increase in chromosome aberrations. Nitroso-IMI is not clastogenic in this system under these conditions.	Heidemann, 1989
CHO-K1 Cells (nitroso TP)	• doses, -/+ S9: 0, 0.25, 0.5 and 1 mg mL^{-1} • exposure time: -S9, 24 and 48 h +S9, 4 h • chromosome aberrations	Possible slight increase in % cells with chromosome aberrations under -S9 condition.	Usami *et al*., 1988
Primary Hepatocytes of Male Rats (nitroso IMI)	• 0.04, 0.13, 0.44, 1.33, 4.44, 13.33, 44.44, 133.33, 444.44, and 1333.33 mg mL^{-1} • 18 h exposure; 3 replicates	Severe cytotoxicity observed only above 133.33 mg mL^{-1}.	Fautz, 1989
Mouse fibroblast M10 cells (desnitro-IMI)	• 3 day incubation • up-regulation of α4β2 AChRs and mechanisms of up-regulation	Desnitro-IMI TP gave up-regulation by eightfold and EC_{50s} of 870 and 500 nM, respectively. Determinated IC_{50} for densitro-IMI (8.2 nM).	Tomizawa and Casida, 2000
Mouse neuroblastoma N1E-115 cell line (desnitro-IMI)	• 30 min (0-180 min) incubation (0.0001-1000 μM) • ERK kinase cascade • intracellular calcium (Ca^{+2}) moblisation	Desnitro-IMI induced ERK activation in a dose-dependent manner triggered by primary action at the α4β2 nAChR with an involvement of intracellular Ca^{+2} mobilisation.	Tomizawa and Casida, 2002

To note: Studies performed by Usami *et al*., 1988 and Fautz, 1989 were evaluated as "*unacceptable, but possibly upgradeable*" in CDPR report (2006).

1.5. Biological systems: *in vitro* and *in vivo* models

Adequate and reliable data regarding adverse effects of exposure to chemical agents are crucial in order to protect human health and environment. The principal source of this information involves often the use of laboratory experimental animal and associated tests. For ethical, scientific and economic reasons, over thirty years there has been present an intensive debate and research how to **reduce**, **refine** and **replace** these animal tests (**3Rs**), without compromising the high level of human health and environmental protection demanded by the European Community. For these purposes in October of 1991 was created the European Centre for Validation of Alternative Methods (ECVAM), pointing to a requirement in the Directive 86/609/EEC on protection of experimental animals used for scientific purposes. The Directive 86/609/EEC was subsequently revised and replaced by the currently active Directive 2010/63/EU which has further enforced the role of ECVAM. In addition, the implementation of the new EU chemicals Regulation REACH (EC, 2006) and the 7th Amendment of the Cosmetics Directive (EC, 2003) motivated the community for development of easier and scientifically more reliable alternative toxicity tests.

In order to understand the mode of action and interactions of pesticides in humans or animal organisms we have to rely on ***in vitro* techniques** which are relevant for the development of sensitive eco-toxicological endpoints. ***In vitro* bioassays** as simplified model systems can, to a certain extent, replace toxicity tests on whole organisms and can be used to test the toxic potential of chemicals or contaminated environmental samples. *In vitro* bioassays based on specific cellular mechanisms measure cellular toxic effects with the use of cell cultures or transgenic bacteria or yeast. Cell cultures provide the finest system for studying toxic molecular mechanism, by permitting the cells to be studied in a monitored and isolated environment, which regulates their activities. In general, the major advantage of cell line usage is the reduction of the complexity of study systems, replacement of animal tests (**3Rs**) and offering benefits in terms of ethical consideration. Mammalian cells belong to tissues, organs and specialised cell types (e.g. neurons) and therefore have a substantial level of internal spatial complexity or cellular activity which results in definite molecular mechanisms. Primary or stable cell cultures maintain their

intrinsic cell-tissue molecular characteristics and therefore can provide tissue-specific and potentially useful informations on pesticide exposure toxicology. Often is impossible to transfer obtained mouse/rat facts to effects on humans, so the use of human-derived cell cultures may offer additional data. However, a major problem of *in vitro* systems is that they typically cannot take in consideration species-specific differences in sensibility and make difficult to extrapolate obtained results to whole organism models. It is often mentioned in literature that the use of *in vitro* models is 'a compromise between convenience and relevance' (Brandon *et al.*, 2003, Pelkonen *et al.*, 2005; Abbas, 2010).

The understanding of toxicant uptake and responses at whole organisms, not only cell, level has also a high ecological importance and is almost impossible to completely eliminate the need of organism models in ecotoxicology studies. For a better evaluation of integrative effects on whole organisms, validated and standardised ***in vivo* assays** with test species from different trophic levels (bacteria, algae and invertebrates) are applied. The use of standardised and well-established test (e.g. ISO Standard tests) in laboratory experiments is desirable as it allows achieving reproducible and comparable data. These bioassays measure effects on parameters such as growth, mortality, reproduction and feeding activity (conventional endpoints), as well as effects based on more specific biochemical / molecular biomarkers.

In addition, the monitoring of health and environment risks should be integrated with chemical analyses, screening of pollutants and possible TPs production. For example, the accessibility of LC-MS techniques in modern research has revolutionised analytical methods used for the analysis of organic molecules including pharmaceutically active compounds, veterinary drugs and pesticides (Kuster *et al*., 2006). The up-to-date LC-MS instrumentation has excellent sensitivity and allowed the development of methods that permit the simultaneous analysis of at least 50 pesticides in different samples with high efficiency (Alder *et al*., 2006).

Throughout this research a battery of eco-toxicological assays was applied. These assays utilised test organisms and cell models from different taxonomical and organizational level providing a possible comprehensive view of the IMI toxicity by reflecting different species susceptibility to IMI (as pure compound or as commercial formulation) and to its TPs. In this study, the following assays adopting a neuronal

cell line F11 and a bacterial strain *S. typhimurium* were referred to as *in vitro* bioassays related to specific measured endpoints. The term i*n vivo* bioassays was used for assays with luminescent bacteria (*Vibrio fischeri*), algae (*Desmodesmus subspicatus*) and aquatic crustacean (*Gammarus fossarum*).

1.5.1. *In vitro* models applied in this research

Neuronal cell line F11 - in the field of *in vitro* toxicology, *in vitro* techniques based on cellular neuronal line models can be used to analyse the neurotoxic effects of chemical substances such as pesticides and their mechanisms of neurotoxicity. In this way, the cellular, morphological or functional changes analysed by the use of this *in vitro* models can be associated with mechanisms linked to toxic effects. The objective of this *in vitro* test was to use immortalised neuronal F11 cell line derived from a fusion between mouse embryonic neuroblastoma N18TG-2 and rat dorsal root ganglion (DRG) neurons (Platika *et al*., 1985) to better understand IMI toxic mode of action (Fig. 13). In addition, as little toxicity found for IMI in different published studies, we decide to investigate if IMI could sensitise sensory neurons that innervate the skin and that are on of major players of dermal exposure route (likely to occur in occupational toxicity to pesticides). As their origin, DRG are a complex neuronal population constituted by peptidergic and non-peptidergic neurons of different size, expressing different sub-population of neurons generating different molecules. Peptidergic neurons are characterised by a strong exocytosis of many different neuromodulators and inflammatory peptides such as: neuronal growth factor (NGF), brain-derived neurotrophic factor (BDNF), calcitonin gene-related peptide **(**CGRP), substance P, atrial natriuretic peptide, adenosine triphosphate (ATP) and glutamate. Upon depolarising stimuli, sensory neurons are transmitting sensory environmental stimuli to the brain, and may undergo to long-lasting activation that may result in diseases characterised by hypersensitisation of peripheral fibres. Since IMI is a nAChR agonist, it is presumed that in F11 cells can operate acting on α3 subunit of nAChRs, which is highly expressed in this cell line. A detailed characterisation of F11 cell model was performed to evaluate IMI effects on cell morphology/survival, signalling pathways, and to identify additional mechanisms of IMI's action (e. g. IMI-induced oxidative stress). On the F11 cell model were tested the cytotoxic

effects of IMI (as pure compound or as commercial Confidor 200SL) and also all four IMI TPs (6CNA, desnitro-IMI, 5-hydroxy-IMI, olefin-IMI).

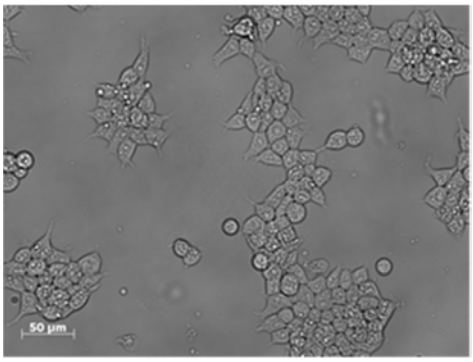


Figure 13: Image of F11 cell line in culture taken by bright field microscopy (20x).

***In vitro* bacterial reverse mutation (Ames) test -** the bacterial reverse mutation test uses amino-acid requiring strains of *S. typhimurium* (Fig. 14) to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in experimental animals and humans. The particular bacterial *S. typhimurium* strain (LT2) with a deep rough mutation *(rfa)* is relatively harmless and is useful in the determination of the possible genotoxic potential of several samples. This strain is used in Ames test, an ISO/DIS Standard 16240 (2005) test and is able to measure mutagenicity of chemicals that induce point or frame-shift mutations. However, it is important to note that the bacterial reverse mutation test utilises prokaryotic cells, which are different from mammalian cells in their uptake, metabolism, chromosome structure and DNA repair processes. Therefore, the *in vitro* test such as this one generally requires the use of an external source of metabolic activation to produce *in vitro* derivatives by liver detoxification enzymes. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions and often does not provide direct information on the mutagenic and carcinogenic potency of tested substance in mammals. Generally, the bacterial reverse mutation test is rapid, inexpensive, and is employed as an initial screen for genotoxic and point mutation-inducing activity.

This method was included in our experimental strategies to explore mutagenic or carcinogenic potential of tested IMI, its commercial formulation and TP (6CNA).

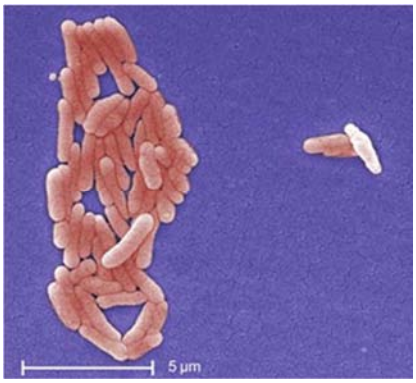


Figure 14: Coloured scanning photomicrograph under a magnification of 5000x shows numbers of clustered Gram-negative* Salmonella typhimurium *bacteria (AP Photo/CDC, Janice Haney Carr).

1.5.2. *In vivo* models applied in this research

The test organisms incorporated in these *in vivo* assays included species of different taxonomic group and different trophic level as microorganisms, plants and invertebrates.

Bioluminescent marine bacteria *Vibrio fischeri* – bacteria play an important role in the environmental fate of applied pesticides and other chemical compounds. For over 25 years the luminescent bacteria test with marine bacterium *V. fischeri* is used as a basic bioassay for eco-toxicological testing of chemicals, waste water and eluents or leachates from soil and sediment. *V. fischeri* is a Gram-negative bacterium with a unique characteristic, bioluminescence, which is controlled by a small set of genes known as the *lux* operon (Fig. 15). The *lux* operon is a 9 kb fragment that consists of genes that code for the subunits of luciferase (*lux*AB) and for enzymes (*lux*CDE) that convert organic compounds to oxidised substrates.

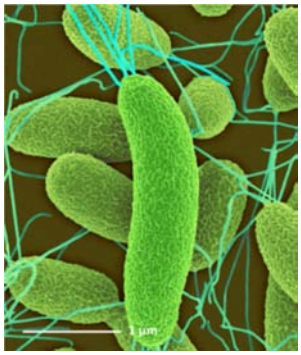


Figure 15: Coloured scanning photomicrograph under a magnification of 8000x of* Vibrio fischeri *bacteria a flagellated, bioluminescent, curved rod-shaped marine prokaryote (Dr. Dennis Kunkel Microscopy, Inc./Visuals Unlimited, Inc.; with slight modification).

Luminescence is a chemical reaction in which the enzyme luciferase oxidises organic compounds, such as long chain aldehyde and reduces flavin mononucleotide, so in this way releases free energy in the form of blue-green light at 490 nm. The reaction that takes place in *V. fischeri* is described schematically in Fig. 16.

The bioluminescence produced by the marine bacterium *V. fischeri* is the basis for ISO standard toxicity bioassay that has been used to assess the toxicity of aqueous solution of known or unknown samples. Differences in the amount of light produced (inhibition of bioluminescence) can therefore be correlated to the organism's metabolism and is proportional to the toxicity of the tested sample. The luminescence is the test criterion which is measured after a contact time of 30 min (between bacteria and tested sample) taking into account a correction factor (C*f*) that is derived from the intensity changes of control samples during the exposure time. The inhibitory effect of the sample on the light emission of *V. fischeri* can be determined as a 30 min EC_{50} value (in case that 50 % of inhibition is not reached is possible to determine also the EC_{20} value - concentration that causes 20% inhibition relative to control). The *V. fischeri* light emission inhibition is measured according to the ISO-Guideline No. 11348-Part 1–3 (2007) as defined in a Standard Operation Procedure (SOP). Measurements can be carried out using freshly prepared bacteria (Part 1), as well as liquid-dried (used in this research; Part 2) or freeze-dried (Part 3) bacterial preparations. The ISO guideline 11348 (2007) recommends testing at least one of the three reference substances (3,5-dichlorophenol, potassium dichromate and zinc sulphate heptahydrate) parallel to each testing to ensure the evaluation of the toxicity test.

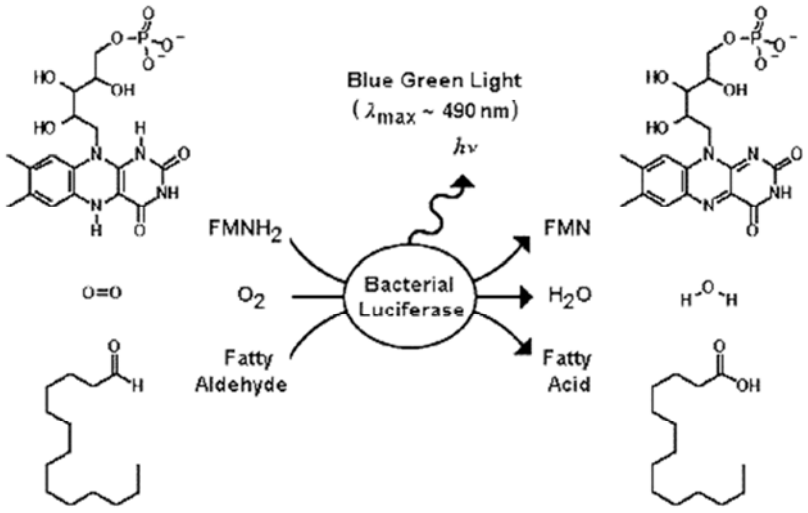


Figure 16: The net chemical equation of the bacterial luciferase catalysed reaction (http://www.photobiology.info/Lin.html).

The effect of IMI on *V. fischeri* was already assessed by Tišler *et al.* (2009) and showed a 30 min IC_{50} of 61.9 mg L^{-1} for IMI and of 56 mg L^{-1} (0.028 % v/v) for Confidor 200SL, while Kungolos *et al.* (2009) determined an IC_{50} of 226 mg L^{-1} for IMI. An additional study was performed by Dell'Arciprete *et al.* (2009) and reported the inhibition of *V. fischeri* luminescence of 32 ± 7 % at concentration 80 mg L^{-1} for IMI. Data relative to TPs include a 30 min EC_{50} of 15.1 mg L^{-1} for 6CNA (Žabar, 2012). The luminescent bacteria was applied as a model during this research to test three more TPs of IMI (desnitro-IMI, olefin-IMI and 5-hydroxy-IMI) which at the moment were not tested for their possible toxic effects towards aquatic organism.

Freshwater green unicellular algae *Desmodesmus subspicatus* - among the potential test organisms, unicellular green algae are commonly used for toxicity tests. It is very important to expand the knowledge about harmful effects of toxic substances in aquatic environment on growth of single-cell algae, since they are an important component of freshwater systems, being the primary producer of organics and oxygen in the trophic food chain (Berden-Zrimec *et al.*, 2008). Any adverse impact on algae is likely to affect organisms at higher trophic levels and may have important consequences for the health status of the whole aquatic ecosystem (Franklin *et al*., 2000). One of the useful ISO/DIS Standard 8692 (2004) test is the freshwater algal growth inhibition test with single cell algae. This test determines the

concentration of contaminant which reduces the growth of microalgae by the means of chlorophyll fluorescence measurements. Test organisms used in this test are planktonic freshwater algae, *D. subspicatus* (Chodat) Hegewald *et* Schmidt (formerly *Scenesdesmus subspicatus* (Fig. 17). This algae species are green algae belonging to the order of *Chlorococcales* (*Chlorophyta, Chlorophyceae*) and are usually unicellular in culture. The recommended strains are available in uni-algal, non-axenic culture from SAG (Collection of Algal Cultures, Germany), ATCC (American Type Culture Collection, USA) or CCAP (Culture Centre of Algae and Protozoa) collections.

Freshwater algae have been used in several toxicity studies (Riva *et al*., 2002; Bengston *et al.*, 2005). Primarily, the measurement of algae growth inhibition of tested samples is used to evaluate the impact of chemicals in aquatic environments. In this respect, unicellular algae *D. subspicatus* were also included in our research to investigate the sub-chronic toxicity of IMI, Confidor 200SL and 6CNA

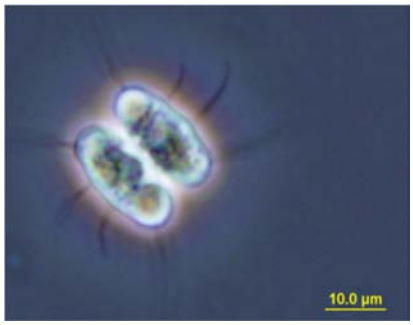


Figure 17: Image of* Desmodesmus subspicatus *algae taken by bright field microscopy (20x).

Freshwater stream scud *Gammarus fossarum* – Invertebrates having simple molecular machinery are often used as good model organisms to explore basic molecular mechanisms occurring in altered physiological states. Particularly, crustaceans are suitable for biomonitoring in aquatic toxicity tests due to their prolific breeding, high abundance in nature and sensitivity to anthropogenic toxic compounds in water bodies which they inhabit (Fernandez-Alba *et al*., 2002). In addition, they are widely distributed, easy to collect and less migratory than fishes.

Amphipoda is one of the most diverse and widespread crustacean's orders. They play a key role in the detritus breakdown process and are commonly used in freshwater risk assessment (Rinderhagen *et al*., 2000; MacNeil *et al.*, 2000).

Moreover, the use of these species is logistically interesting because they can be sampled throughout the year and easily identified, manipulated and maintained/used in the laboratory for ecotoxicological bioassays.

Amphipods (i. e. gammarids) are flattened from side to side and lack of the carapace covering the thorax. They have seven thoracic and six abdominal plated segments, which support and provide shelter for the gills and other soft parts of the animal. The head carries two pairs of antennae, the stalk-less eyes, and the mouthparts (Schram 1989, Barnard and Karaman, 1991). The name 'amphipod' comes, from having seven pairs of walking legs of which the first four reach backwards and the fifth to seventh reach forwards. Typical body length for an adult amphipod is from a few millimetres to a few centimetres, depending on the species. The life cycle of gammaridean amphipods typically consists of five different periods. Since the sexes of amphipods are separate, eggs develop in the brood pouch of the females. Unlike most crustaceans, the amphipods lack a free-living larval stage and juveniles look very much like the adults. Once the juveniles are big enough to start living on their own, they are released. The growth of amphipods is connected to the change of the rigid exoskeleton. After a varying time period and a certain number of moults, juveniles achieve characteristics typical to their sex.

G. fossarum Koch. 1835 is one of most abundant species in freshwater ecosystems in Slovenia (Fig. 18). They are translucent slightly brown benthic animals with alternated periods of swimming and longer periods of burrowing or crawling. Gammarids are often found in high densities in headstreams where they are an important reserve of food for vertebrates, i.e., fish, bird and amphibian species (Friberg *et al*., 1994; MacNeil *et al*., 2000). They are an important component of freshwater ecosystems since they play a key role in leaf litter breakdown process and are important for material transfer in the food web (MacNeil *et al*., 2000; Maltby *et al*., 2002).

Figure 18: Image of stream scud* Gammarus fossarum *(Peter Balej; http://www.biolib.cz).

All these previously described facts explain why amphipods are often used in eco-toxicological studies and why this organism models was included in this study. Furthermore, crustaceans as well as insects belong to arthropods and due to this crustacean-insect relationship they could present a potentially suitable non-target group for IMI testing (Strausfeld, 1998). In general, aquatic biota is extremely variable going from uni- to multi-cellular organisms. Thus for aquatic eco-toxicity testing is essential to use a battery of bioassays representing differences in sensitivity of different test organisms from various trophic levels to cover this variability (Fig. 19).

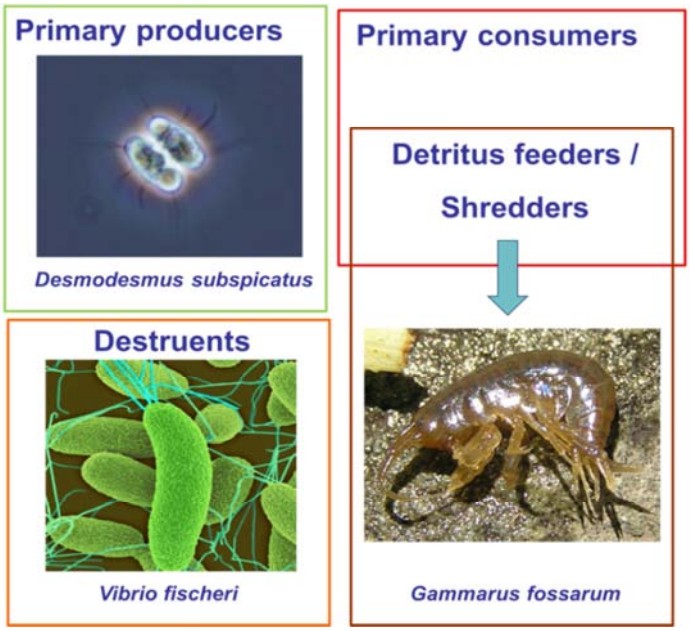


Figure 19: The use of a battery of bioassays involving different model organisms from different trophic levels as an essential tool for predicting adverse effects to the aquatic ecosystem. (Kienle et al., 2011; with slight modifications)

1.6. Biological effects and responses: multi-biomarker approach

The use of molecular biology and biochemical methods to understand toxicity mechanisms provides ecotoxicologist useful tools which permit them to investigate possible **biological effects** or **responses** due to the impact of all pollutants released from both natural and anthropogenic sources in aquatic ecosytems (Carajaville *et al.*, 2000; Sarkar *et al.*, 2006). To analyse such toxicological, biochemical and physiological process is useful to measure specific biomarkers. **Biomarkers** are defined as quantitative measures of changes in the biological system that respond to either (or both) exposure to, and/or doses of xenobiotic substances that lead to biological effects. They show the interaction between biological system and potentially harmful chemical, biological and physical action (WHO, 1993). According to the NRC (1987) and WHO (1993), biomarkers can be subdivided into three classes: **biomarkers of exposure, of effect and of susceptibility.** The use of term biomarker is often restricted to cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism and are indicative of xenobiotic exposure and/or effect changes that occur at the organism, population and more complex levels. One of the functions of biomarkers is to provide early warning signals of biological effects, and that it is generally believed that sub-organismic (molecular, biochemical and physiological) responses tend to precede those that occur at organism or higher levels (Fig. 20).

Different biomarkers were used in order to test potential adverse effects of IMI and its related compounds on model systems described in sections 1.5.1 and 1.5.2 of this manuscript. The selected biomarkers included in this study are described as follows:

IN VITRO MODELS

- **Bacteria *S. typhimurium***
  - mutagenicity potential

- **Neuronal F11 cell line**
  - survival/cytotoxic effects
  - structural/morphological parameters
  - molecular/sub-cellular parameters

IN VIVO MODELS

- **Marine bacteria *V. fischeri***
  - bacterial luminescence inhibition

- **Unicellular green algae *D. subspicatus***
  - rate of algae growth inhibition

- **Amphipod *G. fossarum***
  - mortality rate
  - oxidative stress parameters (e.g. enzyme activity)
  - multixenobiotic resistance (MXR) mechanism
  - behavioural endpoints

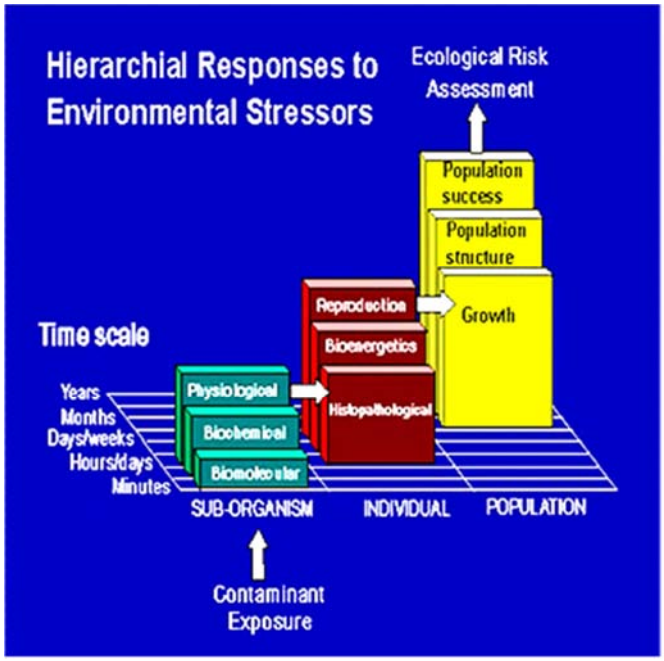


Figure 20: Hierarchical responses to environmental stressors (Adams, 1990).

Biochemical biomarkers (stress enzymes) as indicator of stressful effects

Different classes of **pesticides** may be related to enhanced production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) which could contribute to the toxicity of these compounds (Banerjee *et al*., 2001). Basic cellular metabolism in aerobic organisms involves the production of oxygen free radicals and non-radical ROS (Halliwell and Guttridge, 2007). The imbalance between the generation and the neutralisation of ROS by antioxidant mechanisms within an organism generates the **oxidative stress** (Livingston, 2001; Valvadinis *et al*., 2006) (Fig. 21). Pesticides are known to induce oxidative stress by:

- induction of ROS production as by-products of detoxifying mechanisms,
- alteration of normal mitochondrial respiration processes or
- by their own reduction/oxidation properties.

Pesticide induced oxidative stress can than provoke oxidant-mediated effects (such as increased activities of antioxidant enzymes) and oxidant-mediated toxicities (such as oxidation of lipids, mutations or cell death) (Zama *et al*., 2007; Tebourbi *et al*., 2011). Enhanced ROS are also implicated in pesticide-induced neurotoxicity, based

on their role in biochemical changes that give rise to neuronal cell damage and death (Jia and Misra, 2007). Moreover, oxidative stress induced by pesticides could be one of the sources for neurodegenerative disorders such as Alzheimer's or Parkinson's disease (Bogdanov *et al*., 2001; Huang *et al*., 2004).

The primary line of defence is offered by general antioxidant systems, which have been demonstrated to scavenge ROS and free radicals. There are essentially three systems which include: (i) water soluble reductants (e.g. glutathione, ascorbate), (ii) lipophilic reductants (e.g. α-tocopherol, or β-carotene) and, (iii) enzymes (e.g. catalase, peroxidase or superoxide dismutase). From the point of view of biomarkers, a condition of oxidative stress can be shown through evaluation of specific biomarkers of oxidative damage to macromolecules such as lipids, proteins and nucleic acids (Woo *et al.*, 2006). Generation of ROS and their action affects antioxidant enzymatic activities, lipid peroxidation and mitochondrial function. In addition, oxidative stress by environmental pollutants activates several signalling molecular pathways such as: MAPK pathways or extracellular signal-regulated kinase (ERK) which is involved in cellular apoptosis – cell death (Ki *et al*., 2012).

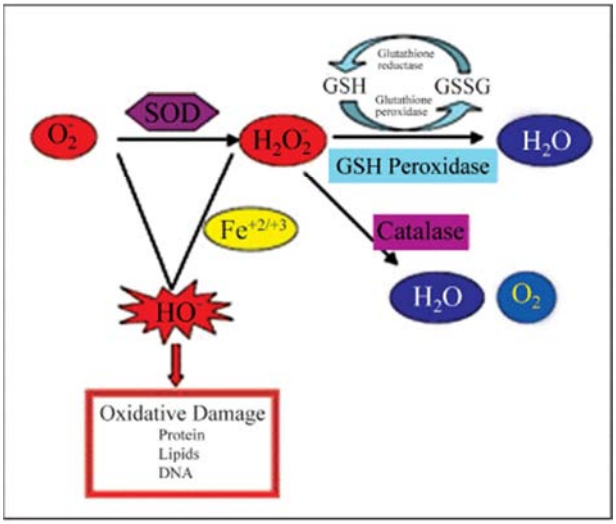


Figure 21: An unbalance between the production of pro-oxidants and antioxidants in cell might lead to strengthened production of ROS which could lead to serious cellular damage (Wakamatsu et al., 2008).

In fact, even if pesticide toxicity is often restricted to their mode of action, as in case of IMI to insect neuronal transmitter receptors, it is important to expand our knowledge regarding other provoked mechanisms (e.g. ROS production) which could contribute to the toxicity of this insecticide. In this research, in order to have a better understanding of the toxic action of IMI and its TPs the involvement of induced ROS production was investigated by measurement of oxidative stress biomarkers such as antioxidant enzyme catalase (CAT), detoxifying enzyme glutathione-*S*-transferase (GST) and the levels of lipid peroxidation (LP) damage in non-target aquatic organism (*G. fossarum*) (Malev *et al.*, 2012) and in neuronal F11 cell line.

CAT is one of the antioxidant enzymes and an essential defence against the potential toxicity of superoxide anions like hydroxyl free radical. CAT extensively decomposes hydrogen peroxide formed during oxidative stress (Winston and Di Giulio, 1991). A change in CAT activity is often an indicator of a cellular damage after exposure to chemicals, and therefore it is considered as an early environmental stress biomarker in different cases. Most studies about CAT activity focus on organisms after exposure to pesticides, hydrocarbon (Brown *et al*., 2004) and heavy metals (Laszczyca *et al*., 2004).

GST belongs to the class of phase II detoxifying enzymes that catalyses the conjugation of glutathione with xenobiotics and cytotoxic aldehydes produced during lipid peroxidation (Banerjee et al., 2001; Bebe and Panemangalore, 2005). GSTs enzymes catalyse the reaction of xenobiotic compounds with the -SH group of reduced glutathione (GSH), thereby neutralising their electrophilic sites and rendering the products more water-soluble (Edwards *et al*., 2000). In addition to catalysing GSH conjugation, GSTs also exhibit glutathione peroxidase (GSH-P_X) activity, which suggests a role in protection against oxidative stress. Consequently, GST activity it is a potential biomarker of a detoxification process induced by very large number of pollutants capable of generating oxidative stress. For example, in insects, GST has been induced by exposure to a number of chemicals, including pesticides, such as lindane (Lagadic *et al.*, 1993), paraquat (Parkes *et al.*, 1993) and oxadiazolone (Hunaiti *et al.*, 1995).

LP is considered an oxidation-related biomarker and it represents the negative effects caused on the lipid membranes by ROS. LP occurs when the natural antioxidant defences are overcome and fatty acid hydro-peroxides formed with the consequence of membrane and membrane-bound enzymes destabilisation (Porter *et al*., 1995; Linden *et al*., 2008). One of the consequences of oxidative stress is the peroxidation of lipids. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds. These include different reactive carbonyl compounds of which the most abundant is called malondialdehyde (MDA) and is used as a marker of LP induced by xenobiotic stressors (Del Rio *et al*., 2005). The most widely used assay for lipid peroxidation is the thiobarbituric acid reactive substances (TBARS) method (Draper *et al*., 1993). MDA as a secondary lipid peroxidation product in biological samples reacts with thiobarbituric acid (TBA) under strong acidic condition and heating. This reaction is actually the formation of a pink-colour product (TBA-MDA) which can consequently be measured by the colorimetric method (Lykkesfeldt, 2007). The detailed reaction is shown in Figure 22.

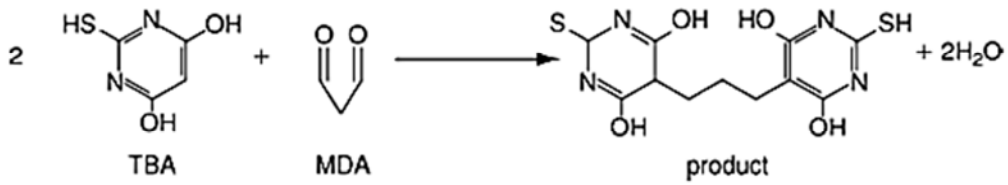


Figure 22: Schematic illustration of MDA-TBA reaction and formation of a colour product (Janero, 1990).

AChE is often used as a biochemical biomarker that indicates stress effects due to pesticide exposure. AChE plays a key role in regulation of cholinergic nervous transmission. It is responsible for the hydrolytic degradation of ACh, which is the primary neurotransmitter in sensory and neuromuscular systems in most organisms. The active site of AChE contains a serine hydroxyl group that binds to anticholinesterase insecticides (with structure similar to Ach), which inhibit the AChE, generating a phosphorylated unreactive enzyme and causing serious damage to neuronal structures. AChE inhibition leads to overstimulation of the nervous system, which results in neurotoxicity and cellular death. Monitoring studies based

on the inhibition effects are majorly related to the presence of organophosphorus insecticides and carbamates (Fulton and Key, 2001). However, evidence of the modulation of esterase activities by other organic chemicals (e.g. surfactants), metals and indirect alteration by neonicotinoids (e.g. IMI) has been described (Jifa *et al*., 2005; Dondero *et al.*, 2010). In our study we tested possible indirect inhibitory effects on freshwater amphipods exposed to IMI (Malev *et al.*, 2012). This measurement was also performed on gills of neonicotinoid exposed mussels and showed an interesting outcome and change of AChE activity (Dondero *et al.*, 2010). In this study was observed a significant inhibition at the lowest and at the intermediate tested IMI concentration.

Morphological, sub-cellular and molecular changes as indicator of stressful effects

In the test that involved IMI testing on neuronal F11 cell model was evaluated not only the final endpoint of **cell survival (cytotoxicity)**, but also sub-cellular and molecular factors which can be evoked upon cell exposure to stressful stimuli. Immunofluorescence (IF) appears as a powerful *in vitro* technique to assess susceptibility of different cell to xenobiotics. Using the IF method is possible for example to detect endogenous proteins induced by different contaminants (Marsili *et al*., 2008) as well as alterations of cellular ultrastructure (Liu *et al*., 1996; Segner and Braunbeck, 1998). In our case was possible to detect the **changes in the cell cytoskeleton** (i.e. actin, tubulin, intermediate filaments) and other ultra-structural alterations. One specifically studied example was mitochondria that are not only an important source of cellular energy but they also preserve intracellular Ca^{2+} levels and regulate calcium dependent cytotoxicity (Foster *et al*., 2006). Various alterations in the number, size shape and **mitochondria membrane potential** can occur in different stressful conditions and can be detected not only ultra-microscopy analysis with (transmission electron microscopy; TEM), but also with the usage of specific molecular dyes (e.g. JC-1) detectable with optical imaging as performed in this research.

Standard high priority targets other than the above mentioned cytoskeleton proteins were also P-glycoprotein. Organisms protect cellular targets via efflux transporters, such as P-glycoprotein (P-gp) and multidrug resistance protein (MRP)

present in cell membranes (Cole and Deeley, 1998, Endicott, 1989). P-gp are ATP dependent transport protein which limit the permeability of xenobiotics into the cell, and recognise or eliminate potentially cyto- and genotoxic metabolites produced during metabolism/detoxification of these compounds (Deeley *et al*., 2006). This system has been termed **multixenobiotic resistance (MXR) mechanism** in aquatic organisms and may be induced or inhibited by a variety of chemicals (Bard, 2000; Kurelec, 1992). MXR mechanism, mediated by the activity of membrane efflux transporters, represents a first line of defence in aquatic organisms exposed to environmental contaminants.

Immunochemical analysis with mammalian monoclonal antibody to cross react with P-gp counterparts has been used to detect the presence of P-gp in aquatic organisms (Sauerborn Klobučar, 2010; Bard *et al*. 2002; Cooper *et al*., 1999). Expression of MXR proteins is often reflective of xenobiotic levels to which an organism is exposed (resistance to environmental toxins is likely conferred by the overexpression of these proteins).

Another common method to detect MXR activity is through **dye exclusion assays**. This assay applies specific fluorescent dyes (rhodamine B, rhodamine 123 and calcein AM) to test the ability of cells to extrude a known MXR substrate. Under normal conditions with fully functional transporters the applied dye is readily expelled from the cells giving low fluorescence values. On contrary, when MXR efflux function is compromised by the use of an MXR inhibitor the fluorescent dye accumulates within the cell (Fig. 23). Dye exclusion assays are relevant for evaluating chemicals, such as environmental contaminants, for their ability to inhibit MXR. This methodology was applied also in this research to investigate the MXR mechanism in aquatic amphipods stressed by neonicotinoid insecticide IMI and its TP 6CNA. In addition was test also a possible synergistic effect of heavy metal (copper) and IMI or 6CNA.

Copper-based compounds are intentionally introduced into the environment as killing agents for fungi and algae. One of the copper based compounds mostly present is copper sulphate ($CuSO_4$) which can be found in most running waters. $CuSO_4$ is in large amounts used as fungicide in agriculture, especially in viticulture as an agent to protect grapes against bacteria and can appear concurrently with IMI in the environment. Copper (as copper oxychloride or copper hydroxide or copper

sulphate tribasic) as foliar fungicide with protective action and IMI as systemic insecticide with protective and curative action are all used in potatoes, tomatoes and numerous vegetable (Tomlin, 2000). It is also used as an algaecide and herbicide in irrigation and municipal wastewater treatment. Trade names of its commercial formulations are Agritox®, Basicap®, and Bluestone®. Copper may be released and become free to catalyse the formation of highly reactive hydroxyl radicals and initiate oxidative damage (Gaetke and Chow 2003; Bopp *et al*., 2008). $CuSO_4$ is water soluble and it doesn't have additional solvent or metabolite that can be toxic to animals. It presents seasonal applications, so very high instant concentrations can be locally observed in freshwaters (de Oliveira-Filho *et al*., 2004). Graves *et al.* (2004) found copper concentrations up to 77.4 mg L^{-1}, which were attributed to the wide use of several forms of Cu as fungicides for tomatoes and direct application of copper sulphate as an algaecide or herbicide in golf courses, lakes and ponds.

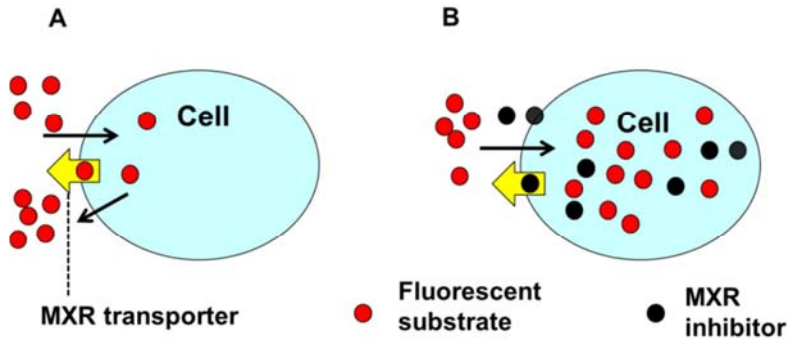


Figure 23: (A) Cell with normal MXR function where transporters recognise the fluorescent dye and expel it from cell which has a low fluorescence value. (B) Cell with disrupted MXR function exposed to an MXR inhibitor or chemosensitizer accumulate the fluorescent dye leading to higher cell fluorescence. (Epel et al., 2008 with slight modifications).

MXR inhibition, can occur when there is an abundance of substrates present, leading to saturation of the efflux system. In this case the pumps become overwhelmed and chemicals are accumulated within the cell (Epel *et al*., 2008). Another way is through chemicals known as "chemosensitizers" (inhibitors, modulators) which inhibit MXR function and thus represent specific environmental contaminants. A wide range of pesticides have been shown to act as MXR inhibitors. Presence of chemosensitizers in the environment may increase the absorption of

xenobiotics and consequently their cellular toxic influence in organisms causing harmful physiological/pathological damage (Žaja *et al*., 2006).

Cells possess adaptive mechanisms to counteract environmental stresses and stimuli that can continuously occur. Usually, these systems may be divided in different categories which include some of the previously mentioned mechanisms such as nucleophilic trapping processes (catalysed by GSTs) or efflux transporters that export toxic compounds or metabolites (MXR). In general, induction of these protective responses requires different components on of which is **nuclear factor (erythroid-derived 2)-like 2 (Nrf2)**, a transcription factor responsible for the induction of downstream genes involved in cell protection from deleterious effects of oxidative stress. Nrf2 target genes encode phase II detoxifying enzymes, antioxidant proteins, ABC transporters, scavenge receptors, chaperone proteins, and so forth (Kobayashi and Yamamoto, 2005). Under normal conditions Nrf2 is sequestered in cytoplasm by a cytoskeletal protein, Keap1. Situation is modified under stressful conditions, when electrophiles and/or ROSs cause dissociation of Nrf2 from Keap1. As a consequence, Nrf2 is translocated to the nucleus where leads to the activation of cytoprotective genes involved in electrophile conjugation, excretion of xenobiotics, ROS scavenging and stabilisation of cellular redox potential. The activation of the Nrf2 – antioxidant response element signalling pathway is a major mechanism in the cellular defence against oxidative or electrophilic stress. Interestingly, mutations in Nrf2 gene, and lack of Nrf2 function in rodents and in humans, has been associated to many chronic diseases (Hayes and McMahon, 2009). Role of Nrf2 transcription factor in cellular response to oxidative stress after IMI exposure was used in this research as an ‘indirect’ marker of IMI-induce oxidative stress.

Molecular changes after the influence of various stressors can induce multiple signalling pathways represented by the one stimulated by **mitogen-activated protein kinases (MAPK)** - ERK, JNK and p38 family members. MAPK are members of a family of serine/threonine protein kinases activated by dual phosphorylation at threonine and tyrosine positions (188 and 190, respectively). These MAPKs are implicated in cellular processes such as gene regulation, metabolic reactions, cell proliferation/differentiation, cell mobility and survival or death (Roux and Blenis,

2004). A thoroughly and comprehensive review the functions of MAPKs is presented by Gerits *et al*. (2007). In general, these kinases are important to elicit signal transduction and nuclear factor activation responsible for controlling the expression of individual genes. To test mechanisms of IMI acute toxicity was evaluated the induction of downstream pathways like p38 MAPK or ERK in F11 cell model.

Mutagenicity parameters as indicator of stressful effects

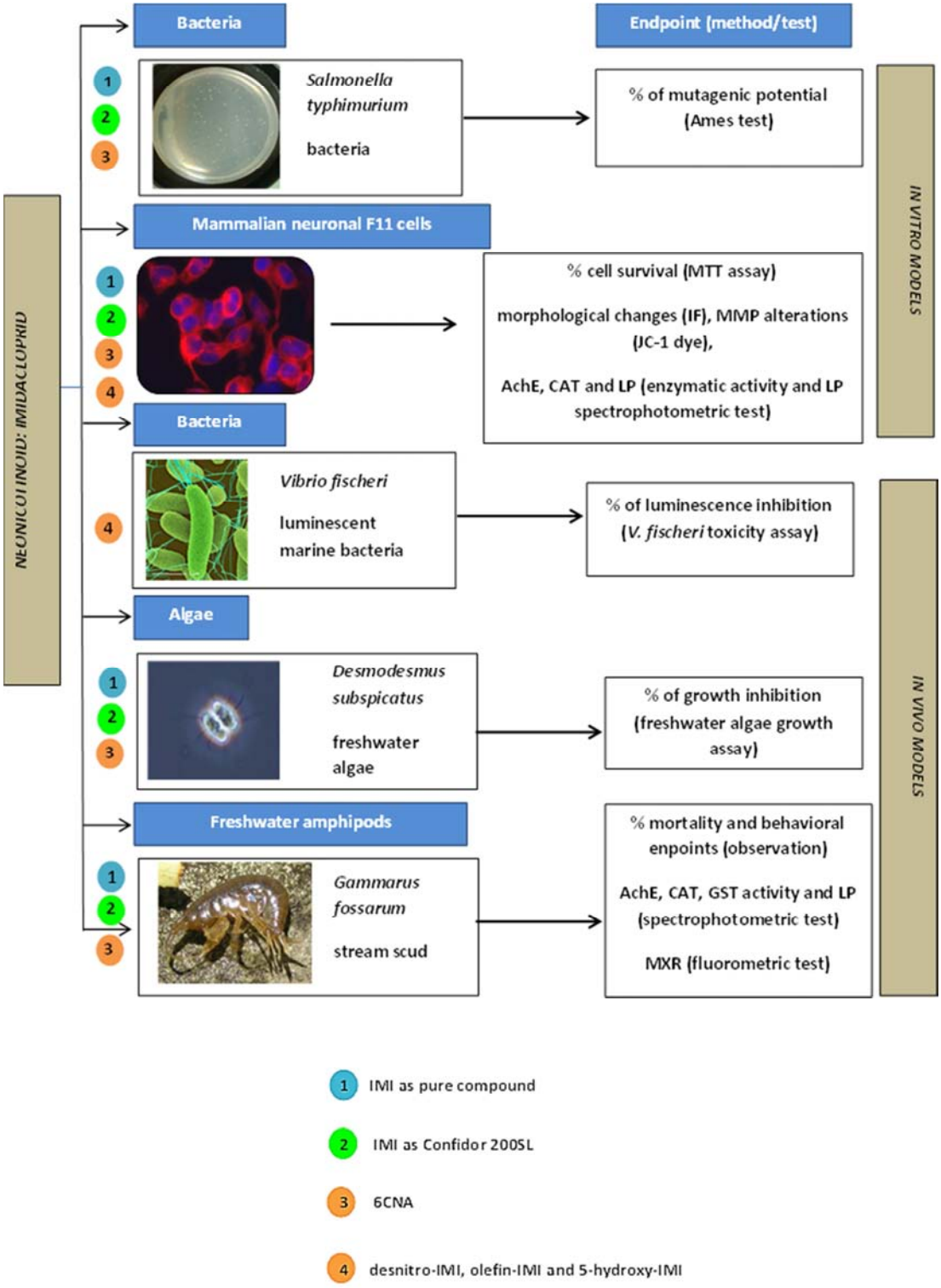
Many environmental chemical pollutants in the aquatic ecosystems have the capability to harm and change genetic material of natural biota. At molecular levels DNA is one of principal site of xenobiotics action (Frenzilli *et al.*, 1999), so the assessment of DNA damage is of primary concern when determining the pollution-related stress in living organisms (Klobučar *et al.*, 2003; Malev *et al.*, 2010; Klobučar *et al.*, 2012). Maintenance of DNA integrity is extremely important for all living organisms, which have developed useful and different mechanisms for protection of genetic material. Genotoxic substances can cause effects such as carcinogenesis, teratogenesis, embryotoxicity as well as a suite of health disorders referred to as genotoxic disease syndrome which is manifested as impaired enzyme function, enhanced protein turnover, impairment immune responses, production of initiators of cytotoxic injuries, inhibition of growth, decreased fecundity and faster ageing that can have adverse effects on stability of ecosystems (Kurelec, 1992). Mutagens are the most dangerous compounds present in ecosystems, because at low concentrations their effect can have consequences at higher levels of organisation that extend for several generations (Bolognesi and Degan, 2001). Furthermore, most chemicals exert their effects via both genotoxic and metabolically toxic mechanisms operating simultaneously. Therefore, there is an increasing need for sensitive assays to monitor the genotoxicity and mutagenicity of the environment. One of these standardised assays called Ames test was also applied in this study to assess the mutagenic potential of the tested neonicotinoid compounds.

Behavioural endpoints

Measurements in this category include assessments of behavioural responses to exposure such as feeding, mating, parental care, and other behavioural modification. Behaviour is considered as a useful tool in ecotoxicology since is one of the early warning indicators of toxicant stress (Pestana *et al*., 2009). During experimental exposure analysed individual biochemical biomarkers should be linked to behavioural responses whenever this is possible (Xuereb *et* al., 2009; Hellou, 2011). A potential advantage of tested behavioural endpoints is in the detection of novel chemicals or chemicals with neurological effects (e.g. IMI), which may provoke more pronounced behavioural changes before effects are detectable at other levels of biological organisation.

1.7. Experimental research design

Figure 24: Schematic diagram of the experimental design with tested models and measured biomarkers after exposure to IMI and its TPs during this research.



1.8. Experimental research objectives

This research project intends to provide a full picture of IMI mode of action and its possible harmful effects for the environment and non-target groups of organisms (biota or human). During this study was proposed the application of a battery of eco-toxicological assays which included bacteria, algae, invertebrates and mammalian cell culture to test the toxicity potential of IMI and it TPs. In addition, these assays were integrated with the measurement of several biomarkers such as biochemical, molecular and physiological responses.

Main objectives of the research project were:

- testing of acute and sub-chronic IMI effects on biochemical, survival and growth parameters in non-target organisms: amphipod *G. fossarum*, unicellular green algae *D. subspicatus* and bacteria *V. fischeri* (a battery of test organisms from different taxonomic and trophic level was selected);
- comparison of IMI effects with effects of its commercial formulation Confidor 200SL and its TPs (6CNA, desnitro-IMI, olefin-IMI and 5-hydroxy-IMI);
- better understanding of the involvement and impact of IMI-induced ROS production and consequent oxidative stress in amphipod *G. fossarum* and in neuronal F11 cells;
- screening of the IMI, Confidor 200SL and 6CNA mutagenic potential;
- investigation of toxic effects of IMI, Confidor 200SL and IMI's four TPs on survival of neuronal F11 cells and its molecular responses discovering the mechanisms with which cells protect and activate stress pathways;
- determination of the utility of *in vitro* cell model systems represented in this study by mammalian neuroblastoma F11 cell line, to obtain information about the specific toxic mode of action of IMI.

The use of a multi-test approach was applied, as it affords a complete screening of the eco-toxicological values of IMI (as pure compound and commercial mixture) and its transformation products.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Chemicals

Tested compounds: IMI was purchased as the Pestanal® grade chemical (99.8 % purity; Sigma-Aldrich, UK), and as a commercial formulation known as Confidor 200SL (200 g L^{-1} of IMI a. i. form Bayer Crop Science Slovenia, Ljubljana, Slovenia). 6CNA was obtained as pure compound (97 %) from Fluka (Sigma-Aldrich, Switzerland). Desnitro-IMI, 5-hydroxy-IMI and olefin-IMI were a courtesy from Bayer Crop Science Germany. Molecular weight in g mol^{-1} of all tested compounds are as follows: IMI (255.66), 6CNA (157.55), desnitro-IMI (247.13), olefin.IMI (271.66) and 5-hydroxy-IMI (253.65).

Chemicals for the HPLC-DAD and LC-MS analyses: acetonitrile CHROMASOLV® and methanol CHROMASOLV® for HPLC grade (Sigma Aldrich), double deionised water (< 18 MΩ cm) prepared through the NANOpure water system (Barnstead, USA) and acetic acid glacial 100 % p.a. from Merck.

Chemicals for total organic carbon (TOC) and total nitrogen (TN) analyses: potassium hydrogen phthalate was purchased from Alfa Aesar GmbH, ammonium sulphate from Fluka, hydrochloric acid 37 % puriss. p.a. from Sigma Aldrich Company Ltd.

Chemicals for biochemical biomarkers measurement: all obtained from Sigma Aldrich: dibasic and monobasic potassium and sodium phosphate, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide, 1-chloro-2,4-dinitrobenzene (CDNB), *L*-glutathione (reduced form), hydrogen peroxide (30%), bovine serum albumin (BSA), Bradford reagent, trichloroacetic acid (TCA), thiobarbituric acid (TBA), malondialdehyde tetraethylacetal (1,1,3,3-tetraethoxypropane) (MDA standard), butylhydroxytoluene (BTH), 96 % ethanol, 1-butanol, hydrochloric acid (37 %).

Chemical for MXR dye exclusion assay: all from Sigma Aldrich: rhodamine B, verapamil hydrochloride, cyclosporine A and phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS).

Chemicals for algal growth inhibition test: standard growth and nutrient medium of the ISO Standard 8692 (2004) prepared according to the protocol using

double deionised water and analytical grade chemicals, potassium dichromate puriss. p.a. (Sigma Aldrich).

Chemicals for Ames test: growth and exposure medium according to the standard protocol ISO 16240 (2005). Additional chemicals all from Sigma Aldrich: 2-aminofluorene (2-AF; Sigma Aldrich), methyl methanesulphonate (MMS 99 %; Sigma Aldrich), *N*-methylpirrolidone, ampcillin sodium salt, citric acid monohydrate, dimethyl sulfoxide (DMSO), β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADP) and *L*-histidine.

Chemicals for bacterial luminescence inhibition test: sodium hydroxide p.a. from AppliChem, sodium chloride from Carlo Erba Reagenti, hydrochloric acid 37 % puriss. p.a. from Sigma Aldrich Company Ltd..

Chemicals used with neuronal F11 cell model: supplied from PAA-Austria: Dulbecco's modified Eagle's medium (DMEM) - high glucose (4.5 g L^{-1}) with *L*-glutamine (with and without Phenol red indicator), foetal bovine serum (FBS) heat inactivated, penicillin/streptomycin 100 x. Purchased from Sigma Aldrich: trypsin-EDTA solution 10 x , poly-*L*-lysine hydrochloride (PLL), thiazolyl blue tetrazolium bromide (MTT salt), 4′,6-diamidino-2-phenylindole dihydrochloride (≥ 98 %; DAPI), *N*-acetyl-*L*-cystein (NAC), mixed tocopherols (vitamin E), Nrf2 antibodies, and phosphospecific p38 (P-p38), while ERK1/2 (P-ERKs) from Cell Signalling Technology Inc. From Life Technologies were obtained: wheat germ agglutinin conjugates (WGA) Alexa Fluor® 488 conjugated lectin, Alexa Fluor® 594 phallodin, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1), SB203580 (p38 inhibitor) and Alexa Fluor® 594/488 anti-mouse or anti-rabbit IgG dyes (secondary antibodies).

All used chemicals were of the highest commercially available grade.

2.2. Analytical chemistry methods: HPLC and LC-MS

Residues of IMI and its TPs were extracted from analysed cell/invertebrate organism samples. Briefly, the samples were processed using a mixture of methanol/water (3:1; v/v) with previous pre-treatment that includes homogenisation or lysis. After extraction the samples were filtered (0.45 μm pore size) and injected into the HPLC, chromatographed under isocratic reversed phase conditions and detected by MS with electrospray ionisation, in the case of LC-MS.

To ensure reliable toxicity data, the stability of IMI and 6CNA was checked. The exposure was confirmed measuring the concentrations of the specific chemicals at the beginning and end of the experimental trial, under the same condition as all toxicity tests (described in Sections 2.3.1; 2.4.2.1 and 2.4.2.2). IMI and 6CNA samples were taken in duplicates and all determinations were performed in three experiments.

IMI and 6CNA were extracted with the use of the miVac centrifugal concentrator Modular Series (Genevac). The water was evaporated (under maintained vacuum conditions at 70 °C for approximately 200 min). The dried leftover was re-dissolved in 500 μL of double deionised H_2O. All prepared samples were stored in glass vials under dark at 4 °C until subjected to HPLC-DAD (UV-Vis). Previous procedure was applied for samples with lower concentration of chemicals (used for tests with amphipods), while samples with higher concentration of chemicals (used for tests with algae and F11 cell) were analysed immediately without pre-concentration step. For quantification purposes, calibration curves were prepared in the appropriate concentration range. The r^2 value of the regression line for IMI was 0.9999 and for 6CNA was 0.9996.

Aqueous solutions of IMI and 6CNA were analysed by HPLC-DAD (UV-Vis) consisting of an Agilent 1100 Series chromatograph, coupled with a DAD detector operating in the UV-Vis range. The separation was achieved using a Zorbax C8 column (250 mm x 4.6 mm) filled with a stationary phase Chromasil 100 (pore size 5 μm, end-capped) produced by BIA Separations d.o.o., Slovenia. The column thermostat was maintained at 25 °C and injection volume was 75 μL. According to Žabar *et al.* methods for IMI (2012) and 6CNA (2011) detection were applied. For IMI detection the eluents consisted of 30 % acetonitrile (A) and 70 % acetic acid

0.75 % v/v (B); isocratic elution; flow rate was 1 mL min^{-1}. The wavelength was 270 nm and the retention time was 8.9 min. While for the 6CNA detection the eluents consisted of acetonitrile (A) and acetic acid 1.5 % v/v (B); flow rate was 1 mL min^{-1}. The gradient elution was as follows: 0–16 min 15 % A; 16–20 min 70 % A. The wavelength was 242 nm and the retention time for 6CNA was 13.2 min.

2.3. *In vitro* models

2.3.1. Neuronal F11 cell line

F11 cell lines were grown in DMEM (4.5 g L^{-1} glucose; with *L*-glutamine) and penicillin/streptomycin (10,000 units mL^{-1} penicillin and 10 mg mL^{-1} streptomycin in 0.9 % sodium chloride), supplemented with 10 % (v/v) of heat-inactivated FBS in a humidified cell culture incubator at 37 °C under 5 % CO_2 as a buffer system.

For cell culture and experimental exposure F11 cells were plated at 1-5 x 10^4 cells/cm^2 (corresponding to 5,000-20,000 cells/well) on different types of tissue culture plates (uncoated 48 or 96-well; based on selected method) in DMEM complemented with 10 % FBS and were grown for 24 h before being used. In 6-well plates F11 cells were plated at 2 x 10^5 cells/cm^2 (70 000 cells/well). For microscope cell imaging cells were plated on plastic Petri dishes (35 mm x 10 mm) with PLL (10 μg mL^{-1}) pre-coated glass coverslips at a concentration of 3 x 10^5 of F11 cells mL^{-1} (initial density of 9000 cells per 12-mm glass coverslip) in DMEM medium complemented with 10 % FBS and were grown for 24 h before being used.

Cells were exposed to vehicle (0.004 % v/v; DMSO) alone as control group and to a.i. IMI, Confidor 200SL, 6CNA, desnitro-IMI, 5-hydroxy-IMI and olefin IMI solutions as exposure groups for 24/48 h. A stock solution of 1 M IMI and 6CNA (255.6 g L^{-1} and 157.5 g L^{-1}), as well of all TPs, was prepared in DMSO (solubility of IMI in DMSO is >200 g L^{-1}) with constant mixing until complete dissolving. The test solutions were prepared by adding an appropriate volume of the stock solution in the culture medium to achieve final exposure concentrations. For all compounds was prepared an equal molar concentration range expressed in corresponding mg L^{-1} values as presented in Table 7. The toxicity of known co-formulants incorporated in

Confidor SL 200 (as negative control – a solution consisting of 38.4 % of dimethylsulfoxide (DMSO), 37.5 % of *N*-methyl-2-pyrrolidone (NMP) and 24.1 % of DMEM in place of IMI) was also tested in the same range as Confidor 200SL (% ; v/v). For Confidor 200SL the final concentrations were corresponding to 0.0001–0.5% (v/v) which contained 0.25–1022.4 mg L^{-1} of a.i. IMI.

In certain experiments, cells were pre-treated with *N*-acetyl cysteine (NAC; 1 mM, 30 min), mixed tocopherols (vitamin E; 1 mM, 30 min) or with the p38 inhibitor SB203580 (10 µM, 30 min), to block possible IMI effects and selected molecular pathways.

Table 7: Selected concentration range for all tested compounds during experiments with F11 cell culture.

µM / mg L⁻¹*	*IMI*	*Confidor 200SL (a.i. IMI; % v/v)*	*6CNA*	*desnitro-IMI*	*olefin-IMI*	*5-hydroxy-IMI*
1	**0.25**	**0.25 (0.0001)**	**0.15**	**0.25**	**0.25**	**0.27**
30	**7.6**	**7.6 (0.003)**	**4.7**	**7.4**	**7.6**	**8.1**
100	**25.6**	**25.6 (0.012)**	**15.7**	**24.7**	**25.3**	**27.2**
200	**51.1**	**51.1 (0.025)**	**31.5**	**49.4**	**50.7**	**54.3**
500	**127.8**	**127.8 (0.06)**	**78.7**	**123.6**	**126.8**	**135.8**
1000	**255.6**	**255.6 (0.12)**	**157.5**	**247.1**	**253.6**	**271.6**
4000	**1022.4**	**1022.4 (0.5)**	**630**	**988.5**	**1014.4**	**1086.4**

* ***To note:*** *in the graphical representation of F11 results all applied concentrations were shown as molar values. Equal molar concentrations were applied for better comparison of data.*

2.3.1.1. MTT cytotoxicity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to measure the amount of metabolically active cells. Indirectly the assay can also determine cytotoxicity and growth rate. The MTT assay is based on the protocol described for the first time by Mossmann (1983) and is based on the conversion of yellow MTT to the purple end-product formazan by succinate-tetrazolium mitochondrial reductase, a system that belongs to the respiratory chain of metabolically active cells (Fig. 25). This assay was optimised for the F11 cell line and used in this study.

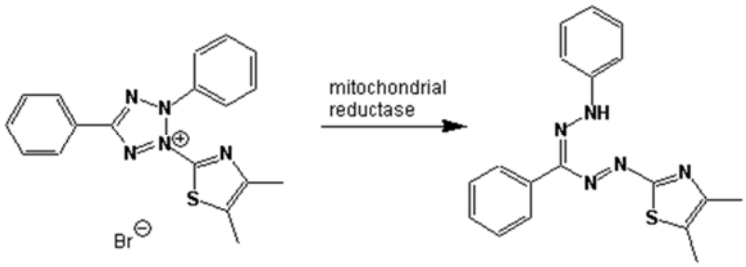


Figure 25: Scheme showing the reduction of MTT tetrazolium salt to formazan (http://en.wikipedia.org/wiki/File:Mttscheme.png).

For testing the cytotoxic effects of IMI and its TPs, assays of viability were performed in 96-well cell-culture plates. Cells were seeded at a density of 3 x 10^4 cells/cm^2 (10,000 cells/well) and cultured for 24 h before being exposed to all tested chemicals in the selected concentration range (Table 7). In addition, only in presence of IMI (1000 μM; 255.6 mg L^{-1} and 4000 μM; 1022.4 mg L^{-1}) for 24 and 48 h, cells were pre-treated with *N*-acetyl cysteine (NAC; 1 mM, 30 min), mixed tocopherols (vitamin E; 1 mM, 30 min) or with the p38 inhibitor SB203580 (10 μM, 30 min).

Briefly, for the purpose of MTT experiments at the end of the exposure in 96-well microplates (for 24 and 48 h) cells were incubated for 2 h with 500 μL of 0.5 mg mL^{-1} of MTT solution in DMEM cell medium without phenol red. After the incubation period, the MTT solution was carefully decanted off and formazan was extracted adding 220 μL of DMSO in each well with gentle shaking for 5 min until complete dissolution was achieved. The plates were read at 550 nm (test wavelength) and 630 nm (reference wavelength) using Tecan F200 Infinite multiplate reader (Tecan Austria GbmH, Austria). Each group was run in 2 or 3 wells and each assay was triplicated (n=9). After analyses the absorbance values and all results were presented as percentage of the control values (viability/control; % of cell survival). Percentage of cell survival and in this case toxicity of the tested compounds is expressed with the following relationship [1]:

% of cell survival =

[Absorbance (570 nm) tested compound / Absorbance (570 nm) control] x 100 [1]

2.3.1.2. Biochemical biomarkers assays in F11 cells

F11 cells were cultured in 6-well plates F11 at 2 x 10^5 cells/cm^2 (70 000 cells/well) in DMEM medium supplemented with 10% FBS for 24 h before addition of the various tested compounds (Table 7). All assays were run in three separated and repeated experiments (n=3), each with two or three replicates.

LP was estimated *in vitro* after the formation of MDA, a major by-product of LP that reacts with thiobarbituric acid (TBA). LP was measured as TBARS release into the culture medium. After pre-culturing, cells were incubated for 24 and 48 h in DMEM medium without phenol red to IMI (1000 µM; 255.6 mg L^{-1} and 4000 µM; 1022.4 mg L^{-1}). Afterwards, the medium was centrifuged for 10 min at 1,000 g and TBARS were measured by mixing the supernatant with 0.7 % TBA and 25 % trichloroacetic acid (TCA) and heating at 90 °C for 10 min. Butylated hydroxytoluene (BTH; 0.5 mM) was included to prevent sporadic LP. TBARS were extracted with addition of 3 mL 1-butanol and the absorbance was measured using the Perkin Elmer Lambda 25 UV/VIS spectrophotometer system at 535 nm and 600 nm, the last one to correct the non-specific turbidity. TBARS were expressed as ng MDA/mg protein using 1,1,3,3-tetramethoxypropane as MDA standard (as fold increase/decrease of control) (Zampieri *et al*., 2009).

CAT activity in cell lysate was determined according to the method of Aebi (1974) and Jamnik *et al.* (2003) by measuring the decrease in absorbance at 240 nm for 2 min due to the decomposition of H_2O_2 in a Perkin Elmer Lambda 25 UV/VIS spectrophotometer system. After exposure period of 24 and 48 h to IMI (1000 µM; 255.6 mg L^{-1} and 4000 µM; 1022.4 mg L^{-1}), the attached cells were washed in PBS (100 mM; pH 7) and lysed in 250 µL of appropriate lysis buffer (50 mM Tris-HCl, pH 8; 150 mM NaCl), scraped and collected in a tube. Cells were sonicated on ice two times for 10 s and centrifuged at 12,000 g for 10 min at 4 °C and the supernatant was collected and used for further analyses. The reaction mixture (2 mL) contained 0.1 mL of cell supernatant in PBS (50 mM, pH 7.0) and 1.9 mL of 10 mM H_2O_2 in PBS (100 mM; pH 7.0). An extinction coefficient for H_2O_2 at 240 nm of 40 L cm^{-1} mol^{-1} (Aebi, 1974) was used for the calculation. The specific activity of CAT was expressed as µmoles of H_2O_2 reduced per minute per mg protein (as fold increase/decrease of control).

All the data relative to biochemical biomarkers are normalised to the **total protein content**. Cell lysate used for the analysis was used also for protein determination. Protein concentration in the cell extract was determined by the Bradford method (1976) using bovine serum albumin (BSA) as a standard. This method is based on the reaction between Coomasie Brillant Blue G-250 (Bradford reagent) and the proteins present in the sample to form a complex. Coomassie Brillant Blue G-250 exists in two different colours forms, red and blue. The red form is converted to blue form upon binding of the dye to the protein. The binding of the dye to the protein is a very rapid process (approximately 5 minutes) and the protein-dye complex remains dispersed in the solution for one hour. During the procedure, 1.5 mL of Bradford reagent was added to 50 μL of supernatant and mixed up in two replicates for each animal. The absorbance of the blue coloured complexes was measured by the spectrophotometer at 595 nm after at least 2 hours. The standards were made with BSA. As blank a mixture of 1.5 mL Bradford reagent and 50 μL of PBS were used.

2.3.1.3. Immunofluorescence microscopy

For microscope imaging F11 cells were cultured in plastic Petri dishes (35 mm x 10 mm) with PLL (10 μg mL^{-1}) pre-coated glass coverslips at a concentration of 3 x 10^5 of F11 cells mL^{-1} (initial density of 9000 cells per 12-mm glass coverslip) in DMEM medium supplemented with 10 % FBS for 24 h before addition of IMI. For analyses of cell morphology after exposure to IMI (1000 μM; 255.6 mg L^{-1} and 4000 μM; 1022.4 mg L^{-1}) the incubation lasted for 24 and 48 h in DMEM medium with or without FBS.

For p38 and ERK induction experiments, exposure to IMI was at 400 μM (102.2 mg L^{-1}) and 4000 μM for 30 min and for a time-course experiment at 5, 10, 30 and 250 min incubation time (4000 μM IMI).

For Nrf2 experiment exposure to IMI was during a time-course experiment (at 10, 30, 60, 120, 240 min incubation time) at 4000 μM concentration. All assays were run in three separated experiments (n=3), each with ten replicates.

F11 cultures were processed for IF staining with standard protocols. F11 cells were fixed in 4 % paraformaldehyde (in PBS 100 mM; pH 7.4) for 20 min at RT.

After washing twice with ice cold PBS, the cells were incubated in blocking solution (5 % BSA, 1 % FBS and 0.1 % Triton X-100 in PBS) for 20 min at RT. After the blocking of paraformaldehyde (PFA) reactive amino residues and unspecific antigens with blocking solution cell glass-coverslips were incubated on a piece of parafilm with primary antibody in 1 % BSA/PBS in a humidified chamber for 1 h. The following specific antibodies were used: neuronal specific β-tubulin III (1:1000, Sigma Aldrich), anti-active phosphorylated p38 MAP Kinase (1:1000, Life Technologies), anti-active phosphorylated ERK 1,2 (1:300, Cell Signaling) and Nrf2 (1:200; Santa Cruz Biotechnology - to test Nrf2 expression, cells were fixed in methanol for 5 minutes at -20 ^{o}C).

After three washing steps with PBS, cells were incubated with fluorescence labelled secondary antibody in 1 % BSA for 40 min at RT in dark. For secondary immunostaining, AlexaFluor 488- or 594-conjugated antibodies (1:500; Life Technologies) were used (secondary staining alone gives no signal). All secondary antibodies are labelled with suitable fluorofores with ex. at 488 nm for green and at 594 nm for red colour. Cellular actin was visualised directly with phallodin conjugated with AlexaFluor594 (1:500, Life Technologies), while membrane integrity was evaluated with *in vivo* labeling with WGA-AlexaFluor 488 conjugated lectin (1:500, Life Technologies). After incubation with secondary antibodies cell coverslips are washed three times with PBS nuclei were counterstained with the use of DAPI, a DNA intercalator (1 μg mL^{-1}; Sigma Aldrich) with ex. at 360 nm for blue colour. As a final step, glass coverslip with cells are mounted with a glycerol-based anti-bleaching solution VECTASHIELD® medium (Vector Labs, UK) to prevent rapid loss of fluorescence during microscopic examination.

Samples were analysed with Axioscope fluorescence microscope (Zeiss) equipped with AxioVision software (version 4.6.2.0; Zeiss). Images were acquired under controlled exposure and excitations below saturation value, and processed for background subtraction and average immunofluoresence intensity quantification with Image J software (Collins, 2007). A minimum of 200 cells were analysed per each experiment.

2.3.1.4. Evaluation of mitochondria through microscopy imaging

Alterations in mitochondrial membrane potential (MMP) were qualitatively assessed with the use of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) (Hardiek *et al*., 2001). JC-1 has advantages over other cationic dyes as it can selectively enter into mitochondria and reversibly change colour from green to red as the membrane potential increases. In healthy cells with high MMP, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On contrary, in unhealthy or apoptotic cells with low MMP, JC-1 remains in the monomeric form, which shows only green fluorescence. JC-1 dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Consequently, mitochondrial depolarisation is indicated by a decrease in the red/green fluorescence intensity ratio (Fig. 26).

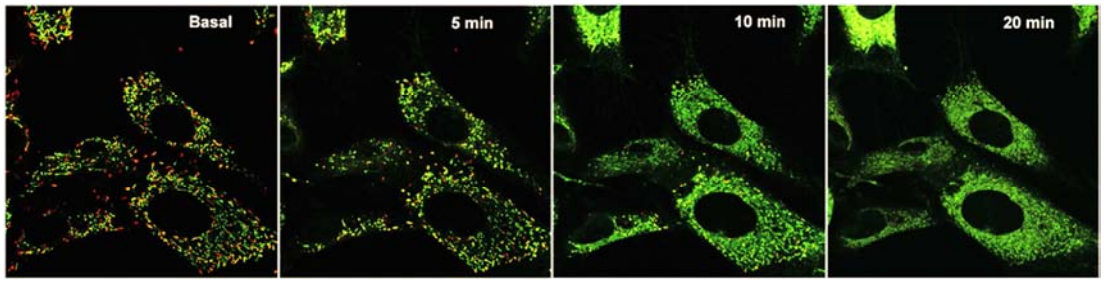


Figure 26: Representative example of JC-1 dye in action. NIH 3T3 fibroblasts stained with JC-1 showing the progressive loss of red J-aggregate fluorescence and cytoplasmic diffusion of green monomer fluorescence following exposure to hydrogen peroxide for 5, 10, and 20 min after treatment (www.invitrogen.com).

For JC-1 *in vivo* microscope imaging F11 cells were cultured in glass-bottom Petri dishes (35 mm x 10 mm) at a concentration of 3 x 10^5 of F11 cells mL^{-1} in DMEM medium supplemented with 10% FBS for 24 h before addition of IMI (4000 μM; 1022.4 mg L^{-1}) and incubation for 15 and 60 min.

A stock solution of JC-1 (200 μM, Life Technologies) was prepared according to manufacturer instructions. Previously prepared JC-1 solution is added (1:1000) to the IMI-treated cells in each well and cells are incubated for 15 min at 37 °C in a CO_2 incubator. After incubation period, cell can be analysed directly in the

culture medium since phenol red does not interfere with fluorescent staining. Healthy cells with mainly JC-1 aggregates can be detected at ex. 560 nm and em. 595 nm, while apoptotic or unhealthy cells with mainly JC-1 monomers can be detected at ex. 488 nm and em. 535 nm. Hydrogen peroxide (100 μM; 30 min), was used as a positive control for decreased MMP.

Samples were analysed with Axioscope fluorescence microscope (Zeiss) equipped with AxioVision software (version 4.6.2.0; Zeiss) with appropriate fluorescence settings. Images were acquired under controlled exposure and excitations below saturation value, and processed for background subtraction and average immunofluoresence intensity quantification with Image J software (Collins, 2007). A minimum of 200 cells were analysed per each experiment. JC-1 assay was run in three separated and repeated experiments (n=3), each with ten replicates. Fluorescence values were expressed as the ratio of fluorescence intensity of J-aggregates (red) to fluorescence intensity of monomers (green), which is often used as an indicator of cell health status. Furthermore, ratio results were presented as fold increase/decrease of the control values (JC-1/control in arbitrary units – AUs).

2.3.2. Ames mutagenicity test with *Salmonella typhimurium*

A set of histidine-requiring strains (TA 98 and strain TA 100) was used for a mutagenicity test called Ames test. Strain TA 98 contains as a marker the frame-shift mutation hisD3052, whereas TA 100 bears the base pair substitution hisG46. Strains are inoculated separately in a Bacto nutrient broth growth medium (20 mL) supplemented with 20 μL of ampicillin solution. Bacteria are grown up overnight at 37 °C under sterile conditions in closed Erlenmeyer flasks permeable to air with caps and mixed by gentle agitation of at least 150 revolutions per minute (rpm). Earlier are also prepared minimal agar glucose plates under sterile conditions.

Test solutions of IMI, Confidor 200SL and 6CNA were prepared at 30; 100 and 1000 μM concentration corresponding to 7.6; 25.6 and 255.6 mg L^{-1} for IMI and Confidor 200SL and 4.7; 15.7 and 157.5 mg L^{-1} for 6CNA. These concentrations are equivalent to: 0.053; 0.178; 1.78 μg/plate for IMI and IMI a. i. in Confidor 200SL, 0.265; 0.89; 8.9 μg/plate for Confidor 200SL (whole formulation) and 0.033; 0.11; 1.11 μg/plate for 6CNA, which is the usual concentration representation in Ames

test. All test solutions are sterilised by filtration through a sterile filer with 0.45 μm pore size. IMI and 6CNA were dissolved in DMSO, which was used at a final concentration of less than 0.004 % (v/v).

For the mutagenicity assay, the control and tested sample solution were mixed with bacterial strains (TA98 or TA100), S9 fraction and 2 mL of sterile top agar (0.6 % agar and 0.5 % NaCl containing 0.5 mM histidine and 0.5 mM biotin) and poured onto previously prepared minimal glucose agar plate [1x Vogel–Bonner salts (0.2 g L^{-1} magnesium sulphate, 2 g L^{-1} citric acid monohydrate, 10 g L^{-1} di-potassium hydrogen phosphate, and 3.5 g L^{-1} sodium ammonium phosphate), 2 % glucose, and 1.5 % agar]. By adding the S9 fraction (homogenate of rat live) directly to the Petri plates it is incorporated in the *in vitro* test an important aspect of the metabolism, making this test highly efficient (Ames *et al*., 1975). S9 treatment for enzyme induction and preparation of the S9 fraction was performed according to the guideline of ISO 16240 (2005). Arranged plates were then incubated in dark at 37 °C for 48 h. Revertant colonies appeared on a background lawn of bacteria. Spontaneous revertants in case of strain TA 98 are around 40, while for strain TA 100 near 100 colonies. Two independent experiments were conducted; each experiment consisted of three replicate plates for each treatment. All steps were performed under sterile conditions.

2.4. *In vivo* models

2.4.1. Toxicity test with luminescent bacteria *Vibrio fischeri*

The toxicity tests with luminescent bacteria were performed to determine of 30 min EC_{50} values for aqueous solutions of three TPs of IMI. The toxicity of desnitro-IMI, olefin-IMI and 5-hydroxy-IMI (at initial concentrations of 100 mg L^{-1}) was assessed using liquid-dried luminescent bacteria *V. fischeri* NRRL B-11177 with system LUMIStox, Dr. LANGE determining reduced luminescence emission after incubation with tested chemicals. Before analyses all samples were adjusted for pH at 7 ± 0.2 with hydrochloric acid or sodium hydroxide solutions and added with 2 % w/v of sodium chloride salt in order to avoid possible adverse effects due to an improper pH value or unsuitable sodium chloride concentration. All luminescence values were measured with a photomultiplier LUMIStox 300 luminometer at controlled 15 ± 1 °C. The toxicity test was performed in three essential steps: (1) an aliquot containing *V. fischeri* was added to each vial in two parallels and luminescence was measured immediately; (2) tested samples were added to vials (at various geometrical dilution levels) with bacteria and regulated to 15 ± 1 °C for 30 min; (3) the luminescence of bacteria within the sample was again measured after 30 min of exposure. All dilution levels were achieved following ISO 11348-2 standard guideline (2007). The blank test was performed with 2 % w/v sodium chloride solution.

The 30 min EC_{50} was calculated with 95 % confidence limits, according to ISO 11348-2 standard (2007) using a computer software supported model. The results are presented as luminescence inhibition in percentage and consequently, the 30 min EC_{50} or EC_{20} values for desnitro-IMI, olefin-IMI and 5-hydroxy-IMI were determined. This way of presenting the toxicity results is commonly adopted in the literature (Sakkas *et al*., 2004; Dell'Arciprete *et al*., 2009, 2010; Kitsiou *et al*., 2009). In addition, percentage inhibitions obtained in each experiment were converted to gamma values according to first model (Jennings *et al*., 2001), where:

$$\textbf{Gamma (Γ) = \% inhibition / (100 - \% inhibition)} \quad [2]$$

Gamma values were plotted against their corresponding chemical concentrations, after first converting all data to natural logs (Ln), to generate Ln gamma/Ln concentration curves for each chemical. In this model, values falling within the 10-90 % inhibition range were used to fit a straight line to the Ln-transformed data by linear regression and liner regression equations have been used to calculate EC_{20} or EC_{50}. To note: EC_{50} value is given by the point of intersection with the x-axis at $\Gamma = 1$, while EC_{20} at $\Gamma = 0.25$.

2.4.2. Green microalgae and crustacean amphipod as test organisms

D. subspicatus (Chodat) Hegewald et Schmidt (formerly *S. subspicatus*) was kindly provided by the Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany. Microalgae were grown in a medium recommended by standard guidelines for freshwater algal growth inhibition test (ISO 8692, 2004).

G. fossarum were collected in April–July 2011 using a net (by the kick sampling method) from the stream Vogršček (Slovenia). The sampling site is in the lower Vipava Valley in Goriška region of Slovenia (45°90’ N; 13°70 E). It is a small waterbed free of industrial, agricultural contamination or human activities and it can be considered unpolluted. This site has a good water quality according to ARSO data record (Agencija Republike Slovenije za okolje-Slovenian Agency for Environment; http://www.arso.gov.si/vode/poro%C4%8Dila%20in%20publikacije/povrsinske_letna.html) and high densities of gammarids were found.

All water samples from the sampling site and during the experimental trial were monitored for temperature, pH, conductivity, oxygen concentration and saturation with a multi-meter WTW350i (with microelectrode replacements for small volumes). In addition, TOC and TN were measured in water samples from the sampling site with a TOC Analitik Jena multi N/C 3100, calibrated with potassium hydrogen phthalate. Before being processed for the TOC and TN analyses, samples were acidified to pH 2–3 with hydrochloric acid.

Gammarids were kept during an acclimatisation period of at least 14 days in a 20 L glass aquarium supplied with thoroughly aerated original stream water. An 8/16 h light/dark natural photoperiod was maintained with the temperature at 12 ± 2 °C in a temperature and humidity controlled chamber and regular water renewal every two

days. Animals were fed *ad libitum* twice a week using a pinch of dry food (e.g. TetraMina® flakes) or raw peas.

2.4.3. Algae toxicity test

Sub-chronic toxicity of pesticides was conducted in 96 microwell plate. The algal inoculum was taken from an exponentially growing pre-culture and added into 25 mL of growth media in order to obtain an initial cell density of 10^4 cells mL^{-1}. Final volume of each well was 200 μL. Serial dilutions of tested pesticides were made in culture medium. Six replicates of controls (untreated) and three replicates of each test concentration were applied. All the plates with cover, control and treatments, were incubated for four days (96 h) at a temperature of 23 ± 1 °C and light intensity of 1100 lux. Algal growth was detected fluorometrically in intervals of 24 h over a period of 96 h in order to achieve a virtual kinetic data distribution. Analyses of chlorophyll fluorescence were performed by a Tecan Infinite® 200 PRO (Männedorf, Switzerland). Measurements were conducted using fluorescence ex. of 440 nm and by an em. of 680 nm. Before reading, tested microplates were shaken for 30 s at 100 rpm. Average of specific growth rates were calculated and subsequently used for calculation of percentage inhibition in comparison to control (Kaczala *et al*., 2011). IC_{50} at 96 h (inhibition concentration that cause 50 % inhibition of algal growth) was estimated for tested compounds using linear regression analysis (Tišler *et al.*, 2009).

Solution of 1 M IMI and 6CNA was prepared in DMSO (solubility of IMI in DMSO is >200 g L^{-1}). Afterwards, a 10 mM (2.55 g L^{-1} for IMI and 1.57 g L^{-1} for 6CNA) stock solution was prepared by the addition of IMI and 6CNA (1 M) or Confidor 200SL to standard algal medium, with constant mixing until complete dissolving. The test solutions were prepared by adding an appropriate volume of the stock solution in the algal medium to achieve final concentration. For all compounds was prepared an equal molar concentration range expressed in corresponding mg L^{-1} values as presented in Table 8. For Confidor 200SL the final concentrations were corresponding to 0.003–0.12% (v/v) which contained 7.6–255.6 mg L^{-1} of a. i. IMI. The toxicity of known co-formulants incorporated in Confidor SL 200 (as negative control – a solution consisting of 38.4 % of DMSO, 37.5% of NMP and 24.1% of

double deionised water in place of IMI) was also tested in the same range as Confidor 200SL.

***Table 8: Selected concentration range for all tested compounds during experiments with algae* D. subspicatus.**

*µM/ mg L^{-1}**	*IMI*	*Confidor 200SL (a.i. IMI; % v/v)*	*6CNA*
30	**7.6**	**7.6 (0.003)**	**4.7**
100	**25.6**	**25.6 (0.012)**	**15.7**
200	**51.1**	**51.1 (0.025)**	**31.5**
500	**127.8**	**127.8 (0.06)**	**78.7**
1000	**255.6**	**255.6 (0.12)**	**157.5**

***To note:** *in the graphical representation of algae toxicity test all applied concentrations were shown as mg L^{-1}values.*

Lower concentrations of IMI than those monitored in this experimental trial were already tested on *D. subspicatus* and showed no effect on algal growth up to 10 mg L^{-1} and due to this fact were excluded (Heimbach, 1986). In addition, as an internal quality control, the bioassays were also performed on the reference chemical potassium dichromate (positive control – $K_2Cr_2O_7$; 0.1–30 mg L^{-1} (ISO 8692, 2004).

2.4.4. Amphipods toxicity test

Gammarids were exposed for 24 h (acute toxicity) to equal molar concentrations of IMI, Confidor 200SL and 6CNA for better comparison. A short exposure period sufficient to promote early alterations (24 h) was used also to mimic runoff-related pulse exposures to pesticides (Pick *et al.*, 1984; Werner *et al.*, 2004). The peak pesticide concentrations usually persist for about 24 h. Furthermore, *G. fossarum* from running water is greatly affected by short-term higher concentration of IMI (Lukančič *et al.*, 2010). Selected sub-lethal exposure concentrations were based on previously determined acute LC_{50} (48 h) and EC_{50} (24 h) values for IMI of 0.8 and 0.07 mg L^{-1} (Lukančič *et al.*, 2010).

Solution of 1 M IMI and 6CNA was prepared in DMSO (solubility of IMI in DMSO is >200 g L^{-1}. Afterwards, a 10 mM (2.55 g L^{-1} for IMI and 1.57 g L^{-1} for

6CNA) stock solution was prepared by the addition of IMI and 6CNA (1 M) or Confidor 200SL to distilled water, with constant mixing until complete dissolving. The test solutions were prepared by adding an appropriate volume of the stock solution in the original stream water to achieve final concentration. For all compounds was prepared, as mentioned, an equal molar concentration range expressed in corresponding mg L^{-1} values as presented in Table 9. For Confidor 200SL the final concentrations were corresponding to 0.000003-0.0002% (v/v) which contained 6.3-511.3 μg L^{-1} of a.i. IMI. The toxicity of known co-formulants incorporated in Confidor SL 200 (as negative control – a solution consisting of 38.4 % of DMSO, 37.5 % of NMP and 24.1 % of double deionised water in place of IMI) was also tested in the same range as Confidor 200SL.

The experimental trial was performed using adult male specimens. After sex determination, total body length (Becuhel and Lønne, 2002) (Fig. 27) and total wet weight was measured (animal were dried between two sheets of filter paper before being weighted). Fifty individuals per exposure concentration were used for every tested compound. Plastic Petri dishes (100 mm x 20 mm; 20 mL volume) covered in order to reduce water evaporation were used for exposure experiments. The bioassays were conducted in darkness, in a temperature and humidity-controlled chamber (12 ± 2 °C; 60 % humidity). After a 24 h exposure period, immobility or moulting and mortality were observed. Live/dead organisms were determined by gently poking and observing movement of appendages. Organisms were counted as dead if none of the appendices were moving after poking for three times. Inactive/paralysed animals were identified when only respiration movements were left (Clesceri *et al*., 1998). Moulted animals were counted based on the presence of the entire old *exuvia* in the exposure vessel (moulted amphipods were not used for biochemical parameters analyses). For each biochemical assay 10 randomly selected gammarids per concentration (from fifty individuals) were processed using whole-body homogenates due to their small body size.

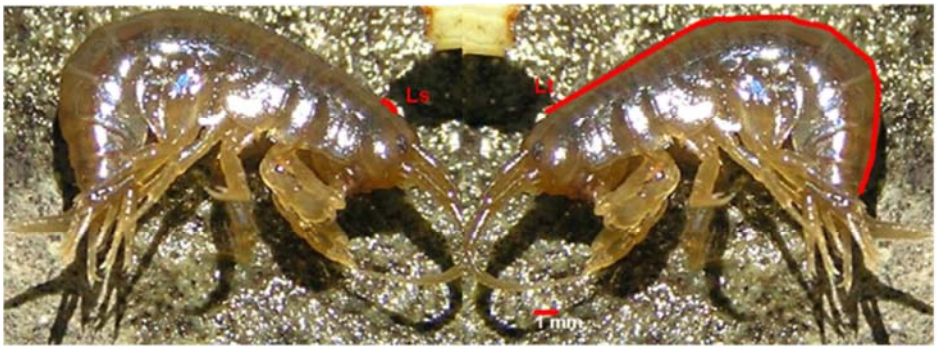


Figure 27: Red lines illustrate the measured length of first pereonal segment (Ls) and total body length (Lt) of* G. fossarum *(Vihtakari, 2008 with slight modifications).

***Table 9: Selected concentration range for all tested compounds during experiments with amphipod* G. fossarum.**

µM/ µg L^{-1}*	*IMI*	*Confidor 200SL (a.i. IMI; % v/v)*	*6CNA*
0.025	**6.3**	**6.3 (0.000003)**	**3.9**
0.05	**12.7**	**12.7 (0.000006)**	**7.8**
0.1	**25.5**	**25.5 (0.000012)**	**15.7**
0.2	**51.1**	**51.1 (0.000025)**	**31.4**
0.4	**102.2**	**102.2 (0.00005)**	**62.8**
0.6	**153.3**	**153.3 (0.00007)**	**94.6**
0.8	**204.5**	**204.5 (0.0001)**	**126.2**
1	**255.6**	**255.6 (0.00012)**	**157.7**
2	**511.3**	**511.3 (0.0002)**	**315.5**

**To note:* *in the graphical representation of amphipods toxicity test all applied concentrations were shown as µg L^{-1}values.*

2.4.4.1. Biochemical biomarkers assays in amphipods

Prior to individual homogenisation, excess chemicals present on the animal's surface were rinsed several times according to Jemec *et al*. (2007). Whole-body specimens were homogenised in 500 µL of ice cold PBS (100 mM; pH 7.0) for 3 min using a glass–glass Elvehjem–Potter homogeniser. The homogenate was sonicated on ice (5–10 s) and centrifuged for 15 min at 3,000 rpm and 4 °C. Freshly prepared clear supernatant was collected and kept on ice to be used for enzyme activities measurements.

AChE activity was determined using DTNB and acetylthiocholine iodide as substrate according to Ellman *et al*. (1961). The principle of this technique is the measurement of the rate of the production of thiocholine (an ACh analogue). Thiocholine, produced during the hydrolysis, reacts with DTNB and the final product of this reaction is a yellow anion of 5-thio-2-nitro-benzoic acid (Fig. 28).

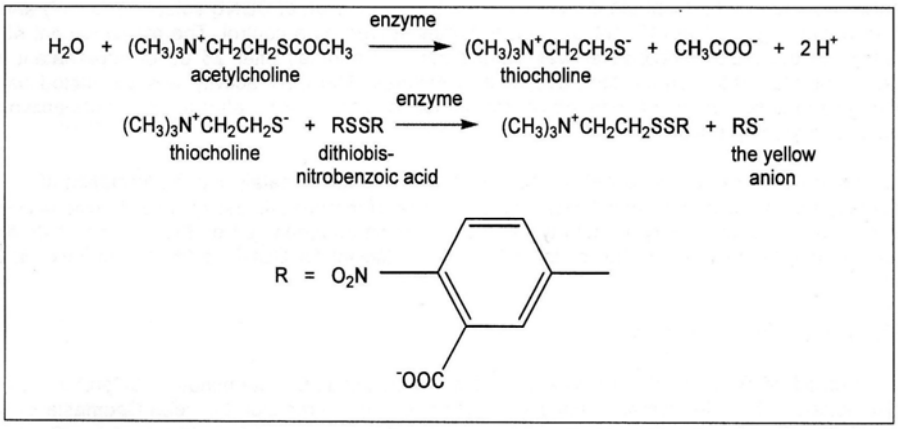


Figure 28: The principle of the Ellman technique (Ellman et al., 1961).

The reaction was followed on a Perkin Elmer Lambda 25 UV/VIS spectrophotometer at 412 nm for 8 min. Kinetic was calculated in the linear range and slope measured (the slope is the rate in absorbance units/min). Spontaneous substrate hydrolysis was assessed using two controls, a blank without ACh and a blank without the sample. The analyses were made in two replicates for each sample. AChE activity is expressed as µmol of substrate hydrolysed per minute per mg protein (based on ε = 13600 L cm^{-1} mol^{-1} for DTNB). The reaction mixture for AChE activity measurement was prepared as presented in Table 10.

Table 10: The reaction mixture used for AChE activity measurement in **G. fossarum.**

G. fossarum
• 2380 μL of PBS (67 mM, pH 7)
• 100 μL of DTNB (0.01 M)
• 500 μL of sample
• 20 μL of acetylcholine iodide (0.075 M)

CAT activity was determined according to the method of Jamnik and Raspor (2003) by measuring the decrease in absorbance on a Perkin Elmer Lambda 25 UV/VIS spectrophotometer at 240 nm for 2 min due to the decomposition of H_2O_2 (ε = 40 L cm^{-1} mol^{-1}). The specific activity of CAT was expressed as μmol of H_2O_2 reduced per minute per mg protein. The methodology and the reaction mixture for CAT measurement are identical as for F11 cells (described in Section 2.5.2.).

GST activity was determined according to the protocol of Habig *et al.* (1974). The method is based on determination of the conjugated product dinitrophenyl-thioether at 340 nm produced from CDNB used as an artificial substrate and reduced glutathione. The conjugation is catalysed by GST isoenzymes (Fig. 29).

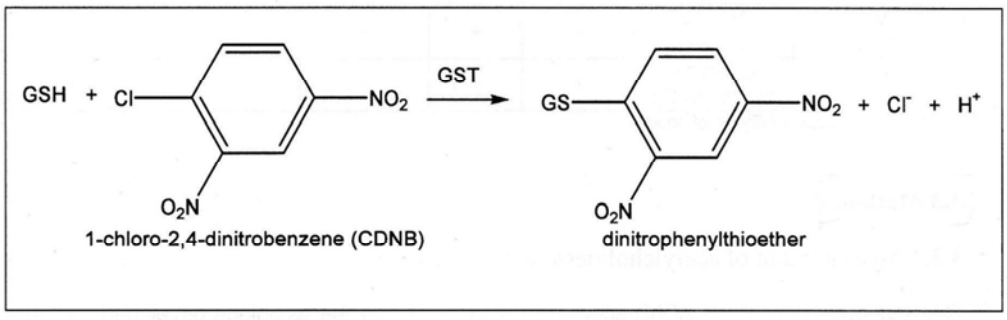


Figure 29: The principle of the GST activity measurement (Habig et al., 1974).

The measurement of the sample was carried out on a Perkin Elmer Lambda 25 UV/VIS at 340 nm for 5 min. The reaction mixture without sample was used as control. Values were expressed as nmol of reduced glutathione and CDNB conjugate formed per min per mg protein (ε = 9600 L cm^{-1} mol^{-1} for CDNB). The reaction mixture for GST activity measure in *G. fossarum* is presented in Table 11.

***Table 11: The reaction mixture used for GST activity measurement in* G. fossarum.**

G. fossarum
• 900 µL of PBS (100 mM, pH 6.5)
• 25 µL of CDNB (60 mM)
• 25 µL of sample
• 50 of reduced glutathione (40 mM)

All the data relative to the enzymatic activity are normalised to the **total protein content** based on the method of Bradford (1976) (as described in Section 2.5.2).

LP was estimated *in vitro* after the formation of MDA, a major by-product of lipid peroxidation that reacts with TBA (Ortega-Villasante *et al*., 2005), with slight modifications. Whole-body gammarids were rinsed, as described previously and homogenised individually in TCA–TBA–BTH reagent [15 % (w/v) TCA, 0.37 % (w/v) TBA, 1 M HCl, and 0.01 % BTH]. Samples were incubated at 90 °C for 30 min, then chilled at room temperature, added 1.2 mL of 1-butanol and centrifuged at 12,000 rpm for 10 min. Absorbance of the supernatant was measured at 535 and 600 nm, the final one to correct for the non-specific turbidity. Before the heating step, absorbance was measured at 280 nm for total protein concentration. These absorbance values of protein content were used to properly normalise absorbance values obtained for LP (Fig. 30).

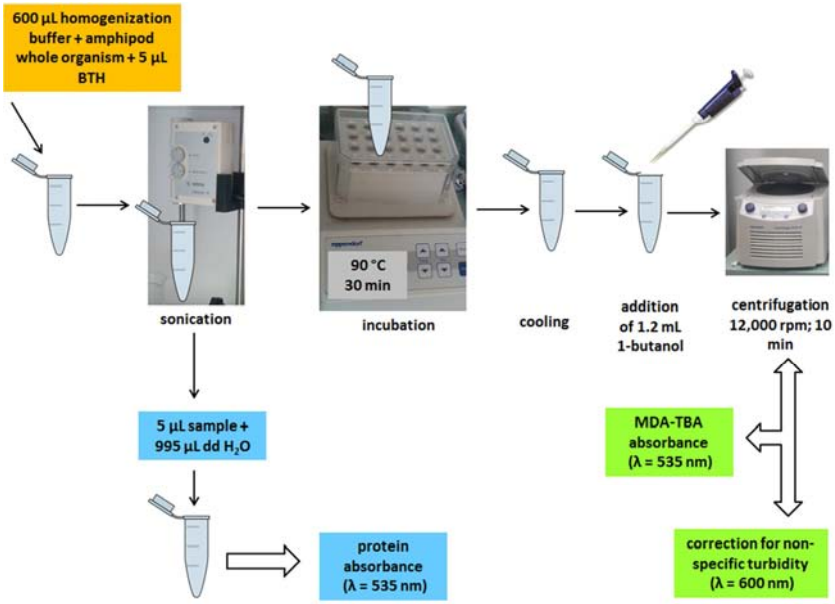


***Figure 30: Schematic representation of lipid peroxidation determination in* G. fossarum.**

2.4.4.2. MXR inhibition assay

G. fossarum was exposed for 1 h to IMI, Confidor 200SL and 6CNA at 3 µM and 30 µM concentration (corresponding to: 0.7 and 7.6 mg L^{-1} for IMI and Confidor 200SL, while for 6CNA to 0.4 and 4.7 mg L^{-1}) and 0.75; 3 and 10 µg L^{-1} copper a.i. During this exposure to tested chemicals was also added a specific model substrate for P-gp called rhodamine B (Rh B). In our preliminary experimental setup the exposure of 1 h resulted in optimal experimental conditions and response.

To test for MXR activity and inhibition, an Rh B exclusion dye assay was adopted. As an indicator of membrane transport activity was used a fluorescent dye called Rh B. Under normal MXR function, amphipods exposed to Rh B will actively expel the dye from cells and the whole-body will have a low fluorescence. On the other hand, amphipods exposed to potential MXR inhibitors are less able to transport Rh B from inside the body, and show increased fluorescence. This increased fluorescence proves the accumulation of Rh B in cells as a consequence of transport activity inhibition. This method was already successfully applied in several studies including aquatic organisms (Smital and Kurelec, 1997; Kurelec *et al*., 2000; Smital *et al*., 2000). In addition, there is also available proof of presence of the MXR system in freshwater amphipods (Timofeyev *et al*., 2007).

Briefly, five specimens of *G. fossarum* were exposed to each concentration of IMI, Confidor 200SL and 6CNA + Rh B (2.5 μM) in Plastic Petri dishes (100 mm x 20 mm; 20 mL volume) covered in order to reduce water evaporation. The assays were conducted for 1 h in darkness, in a temperature and humidity-controlled chamber (12 ± 2 °C; 60 % humidity). Model inhibitors of MXR proteins, verapamil (VER; 10 μM) and cyclosporine A (CA; 10 μM) was run as a positive control alongside all treatments. After 1 h exposure amphipods were removed from their solutions and triple washed in fresh PBS to remove any external Rh B, weighed, transferred to flat-bottom tubes in 0.5 mL of distilled water and homogenised (for homogenisation procedure see Section 2.6.1). The homogenates were centrifuged at 3,000 rpm for 15 min, the supernatants carefully transferred to a 96-well flat-bottom dark microplate (100 μL/well, each supernatant in triplicate), and the fluorescence of accumulated Rh B measured immediately.

The fluorescence of accumulated Rh B in whole-body of amphipods was measured on a microplate fluorometer (Tecan F200 Infinite multiplate reader-Tecan Austria GbmH, Austria) using 535 nm (excitation) and 590 nm (emission) filters. Due to the possible loss of the Rh B-fluorescence intensity caused by the direct exposure to light, glasses, vials/homogenates/supernatants and microplates were light protected with aluminium foil. Fluorescence readings for each well were then normalised to total animal weight and the MXR assay was repeated in three separated experiments.

2.5. Statistical analyses

All statistical tests were performed using STATISTICA 7 StatSoft software. Results from each exposure trial are presented in graphs as mean ± standard error (SE). Statistical comparisons were conducted between control and exposure data using the Student's t-test or the Mann–Whitney rank sum test after the software direct choice of parametric or nonparametric data, respectively. In addition, multiple comparisons were analysed with the One-way ANOVA and Tukey post-test. $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*) were accepted as levels of statistical significance and shown in graphical representations.

RESULTS

3. RESULTS

3.1. *In vitro* model: F11 cells

3.1.1. Imidacloprid cytotoxic effects

Cell viability MTT assay demonstrated limited IMI effects after 24 h and 48 h incubation at the whole micro-molar range of tested concentrations going from 1 μM (0.25 mg L^{-1}) up to 500 μM (127.8 mg L^{-1}) ($p>0.05$, n=9). Slightly reduced but transient effect on cell viability was noticed after 24 h exposure to IMI at 200 μM (51.1 mg L^{-1}) and 500 μM (127.8 mg L^{-1}) concentration ($p<0.05$). Due to the fact that no relevant and permanent adverse effect on lower concentration range was noticed we proceeded with testing even at higher concentrations reaching values of 1000 μM (255.6 mg L^{-1}) and 4000 μM (1022.4 mg L^{-1}).

IMI evoked progressively a dose- and time-dependent lower metabolic activity of F11 cells and reduced survival compared to control especially at concentrations higher than 500 μM (Fig. 31C). Viable cells after 24 h at 4000 μM (1022.4 mg L^{-1}) were of 82.3 ± 3.9 % ($p<0.05$, n=9), while after 48 h at 1000 μM (255.6 mg L^{-1}) and 4000 μM were of 76.2 ± 3.5 % and 56 ± 3.8 %, respectively ($p<0.001$; n=9).

In general, a significant metabolic impairment was found at concentrations larger than 1000 μM accompanied by a significant and progressive cell loss evident by bright field microscopy images (Fig. 31A).

To test F11 morphology after exposure to IMI, a panel of antibodies against cytoskeleton elements such as actin, α tubulin and β type tubulin were applied. In Fig. 32 is shown the morphological characterisation with fluorescence microscopy of F11 cells exposed to IMI (4000 μM) for 24 h that increases the presence of picnotic nuclei (DAPI staining), loss in plasma membrane integrity visualised with fluorescence WGA lectin and strong degradation of cytoplasmic protein components, like cytoskeleton elements (actin and tubulin). In addition to these initial, minor and evident signs of alteration of cytoskeleton, after 48 h IMI induced a drastic re-organisation of the cytoskeleton leading to cytoplasm and nuclear condensation, and finally reducing the overall adhesion of the cells to substrate (Fig. 31A).

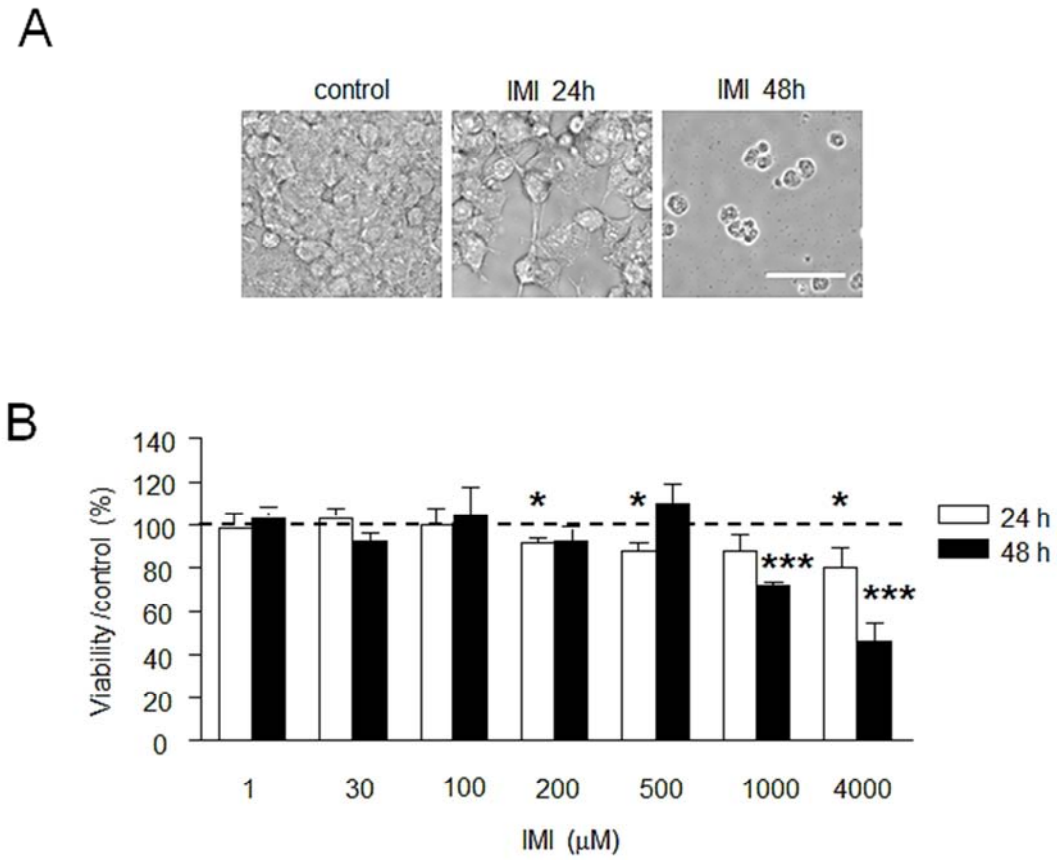


Figure 31: A. Bright field representative images of F11 cells in control and after 24 and 48 h IMI (4000 μM; 1022.4 mg L^{-1}) exposure. Scale bar: 50 μm. B. Values of MTT assay in F11 cells after exposure to IMI for 24 and 48 h; data presented as percentage of control shown as dashed line (n=9). p < 0.001 (***), p < 0.01 (**), and p < 0.05 (*).

Further morphological characterisation of cell culture with serum and serum-free conditions in presence of IMI presented additional alterations. In control conditions cells did not have altered cytoskeleton and any significant signs of stress, while in treated cells there are present modified nuclear features and formation of protein aggregates (Fig. 33A). Also is visualised an interesting differentiation in absence of serum (as expected) with formation of processing and neuronal elongations (Fig. 33B).

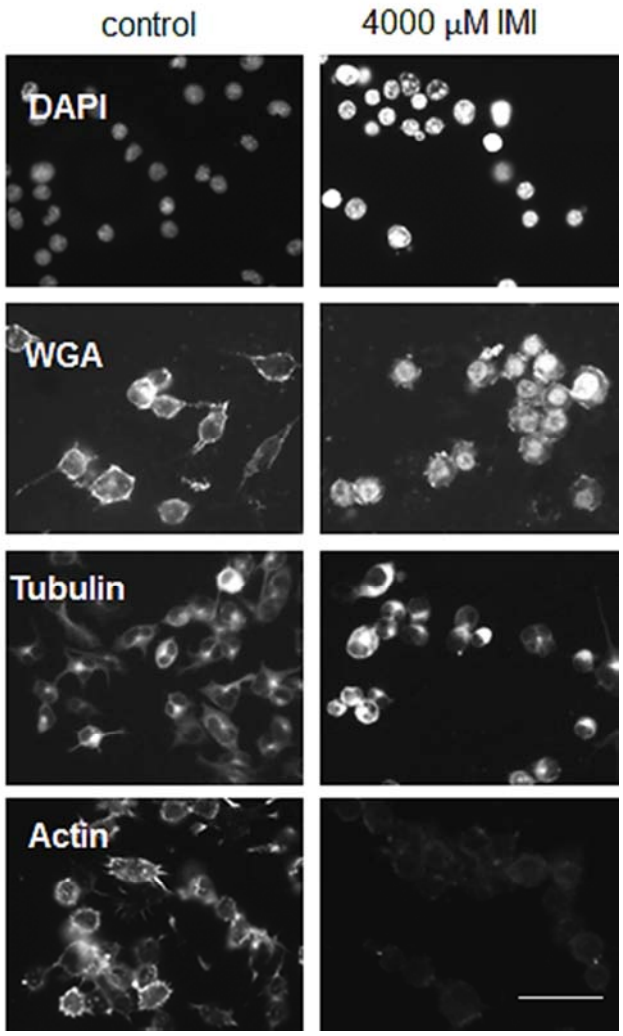


Figure 32: Representative microscopy images of F11 cells morphological characterisation in control conditions and after IMI exposure (4000 µM; 1022.4 mg L^{-1}) for 48 h. Cells were stained with different fluorescent markers, as indicated for each image. To note: stronger nuclear DAPI staining signal after treatment and resilient degradation of cellular proteins (actin staining). Scale bar: 100 µm.

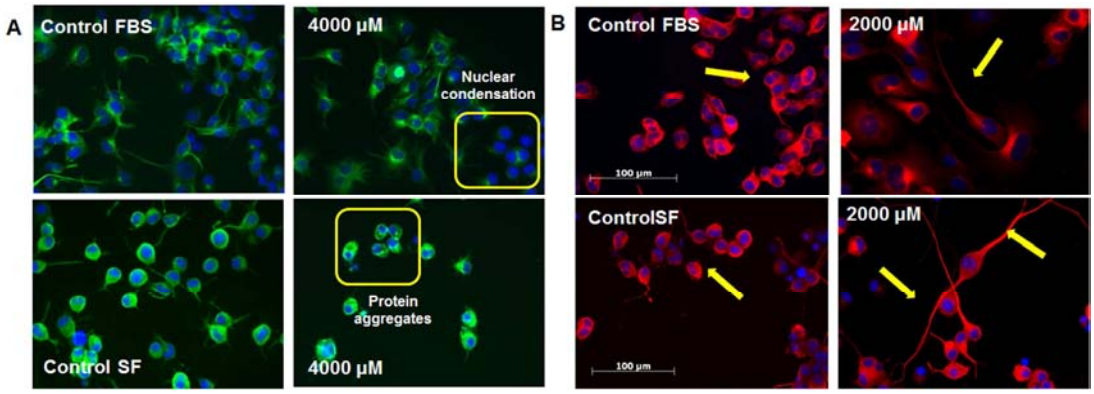


Figure 33: Morphology of F11 cells in control and after IMI exposure (4000 µM; 1022.4 mg L^{-1}) for 48 h in standard growing conditions (FBS) or in absence of serum (SF). A. Nuclei are counterstained with DAPI (blue), β-Tubulin III immunofluorescence staining is shown in green. To note: protein aggregates and nuclear condensation in F11 cells after IMI exposure (yellow rounded rectangles). B. Nuclei are counterstained with DAPI (blue), β-Tubulin III immunofluorescence staining is shown in red. To note: IMI stimulated processes elongation (arrows). Scale bar: 100 µm.

To further cytotoxicity effects of IMI to neuronal cell survival, we compared potentially toxicity of IMI as its commercial formulation (Confidor 200SL - where IMI is the a.i.) and IMI's four TPs: 6CNA, desnitro-IMI, olefin-IMI and 5-hydroxy-IMI.

The negative control (mixture of known co-formulants: DMSO and NMP) did not have any adverse effects on F11 cell at all tested concentrations (data present in Annex section). Due to this fact, all values were compared to control (vehicle only; DMSO). Commercial formulation was found slightly more toxic than parent compound after 48 h of exposure at 4000 µM; 1022.4 mg L^{-1} a. i. (47.8 ± 2.8 %; $p<0.001$ compared to control) (Fig. 34B). Interestingly, 6CNA decreased the F11 cells metabolic activity after 48 h already at 500 µM (157.5 mg L^{-1}) reaching the lowest viability of 72.2 ± 3.5 % ($p<0.01$) at 4000 µM (630 mg L^{-1}). In the case of other IMI's TPs, it is important to emphasise that all significant effects on cell viability were noticed only at concentrations higher than 500 µM for all tested TPs (Fig. 35A and 35B). After 24 h of exposure to 5-hydroxy-IMI at 4000 µM (1086.4 mg L^{-1}) the cell viability was significantly reduced and presented a value of 55.9 ± 2.4% ($p<0.01$). This decreasing of cell viability was significantly enhanced after 48 h at the same concentration for all three TPs. The strongest negative effect and

drastically reduced cell survival at this highest concentration was induced by desnitro-IMI followed by 5-hydroxy-IMI and olefin-IMI. The percentage of viable cells after 48 h exposure to desnitro-IMI (4000 µM; 988.5 mg L^{-1}) was only of 19 ± 2.7 %, while for olefin-IMI and 5-hydroxy-IMI was 24.6 ± 1.04 % and 29.9 ± 2.2 %, respectively ($p<0.001$). These adverse effects were stronger and more evident in case of IMI's TPs compared to both IMI as pure compound or as Confidor 200SL.

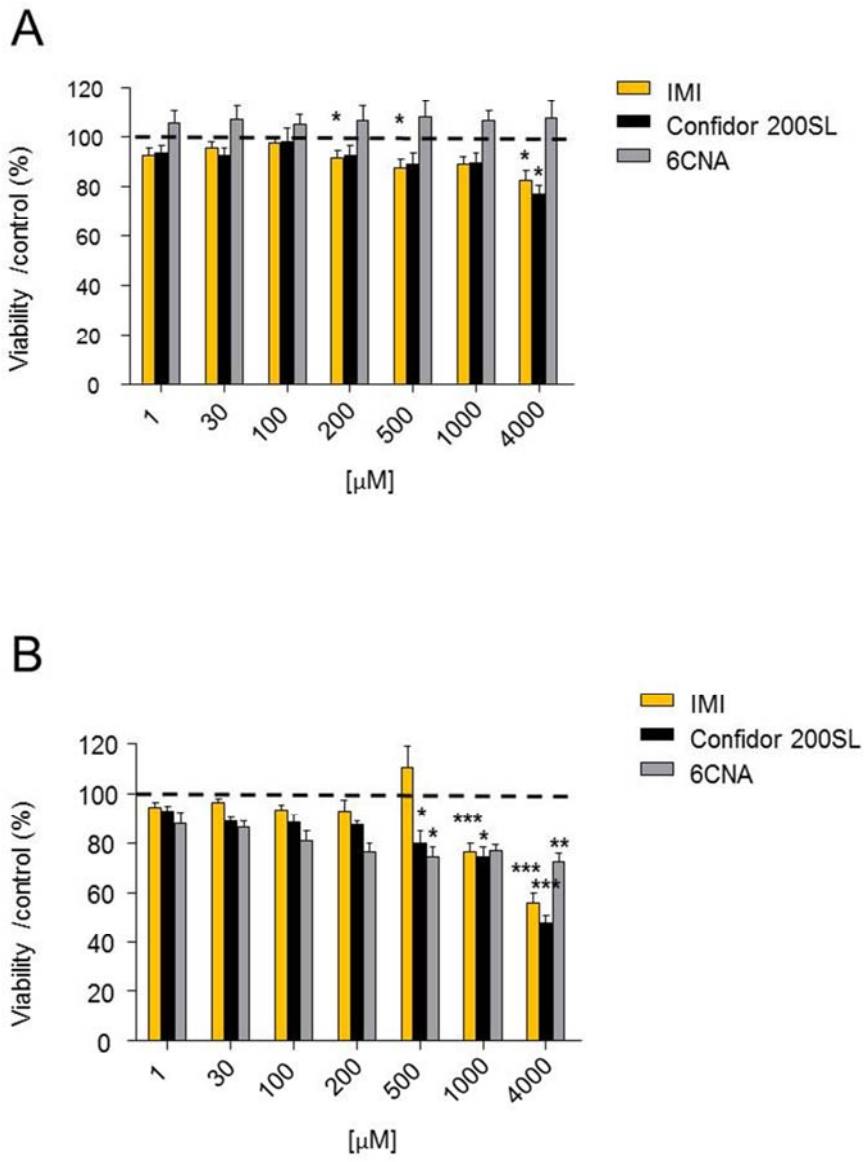


Figure 34: Values of MTT assay in F11 cells after exposure to IMI, Confidor 200SL and 6CNA for 24 h (A) and 48 h (B); data presented as percentage of control shown as dashed line (n=6). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

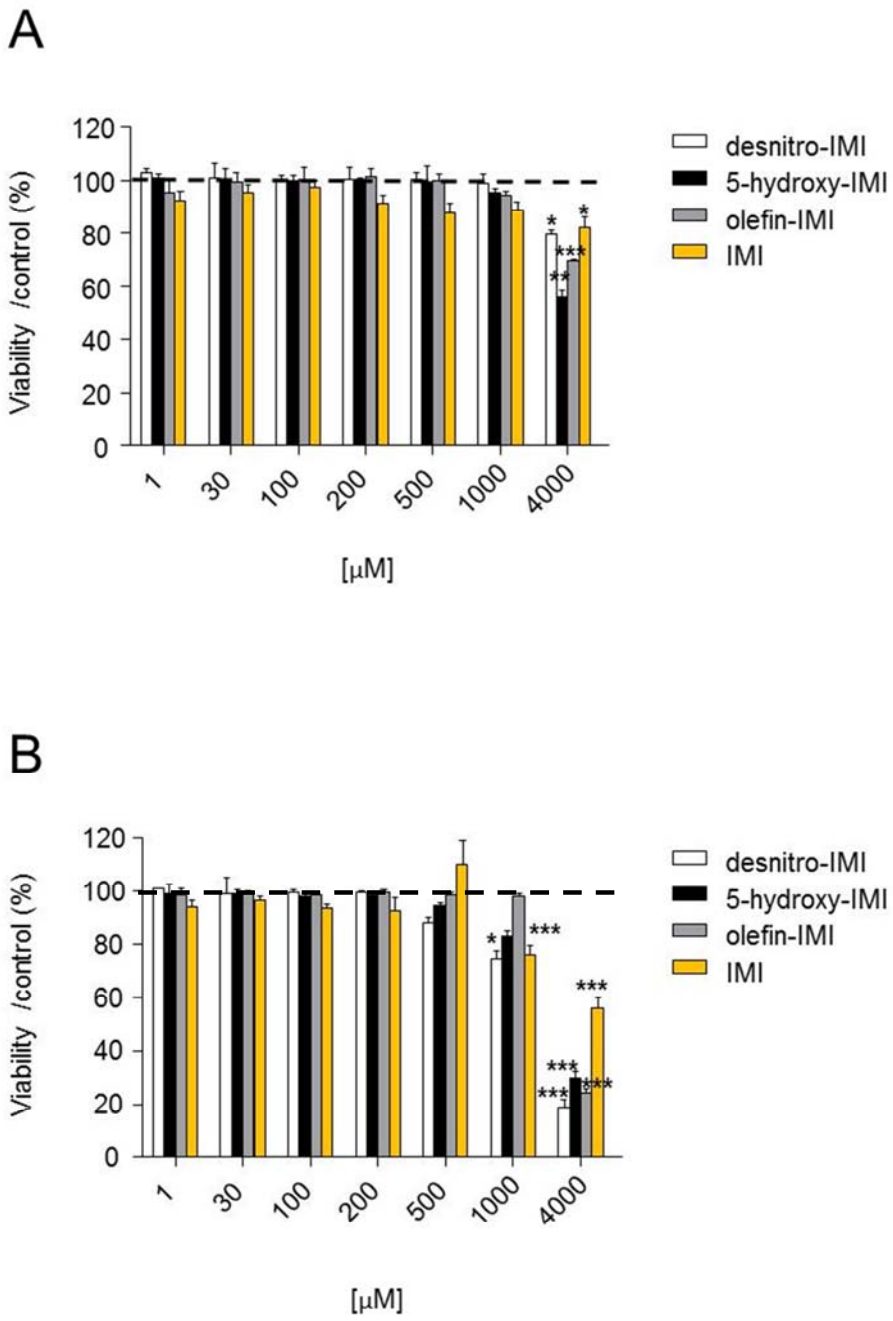


Figure 35: Values of MTT assay in F11 cells after exposure to IMI, desnitro-IMI, 5-hydroxy-IMI and olefin-IMI for 24 h (A) and 48 h (B); data presented as percentage of control shown as dashed line (n=6). p < 0.001 (***), p < 0.01 (**), and p < 0.05 (*).

3.1.2. p38, ERK MAPK and Nrf2 activation in response to imidacloprid

To further investigate the mechanisms of IMI toxicity in F11 cells and if acute IMI exposure was translated into rapid activation of downstream molecular pathways like p38 MAPK and ERK induction, we performed immunofluorescence time-course experiments (0, 5, 10, 30, 240 min) at 4000 μm (1022.4 mg L^{-1}) using specific antibodies against active (phosphorylated) form of kinases ERK 1,2 and p38 (Fig. 36C). In addition, IF experiments were performed on F11 treated cells exposed to different doses of IMI 400 μM (102.2 mg L^{-1}) and 4000 μM (1022.4 mg L^{-1}) for 30 min, using again antibodies that specifically recognise activated form of p38 or ERK MAPK (Fig. 36A and 36B).

The p38 and ERK MAPK were phosphorylated and activated in response to in IMI in F11 cells. These experiments demonstrated that IMI activated these kinases in two different ways. In specific in case of p38 this activation was in a concentration dependent manner with significant induction after 30 min at 4000 μM ($p<0.01$), while in case of ERK a significant and stronger activation response was present after 30 min IMI exposure already at 400 μM ($p<0.001$) (Fig. 36A and 36B). Time course experiments suggested an early activation of p38 MAPK after few minutes (10 min) of IMI stimulation at 4000 μM in the majority of the cells (92.2 ± 1.3 %, $p<0.01$) (Fig. 36C). The p38 activation effect was further quantified and was persistent until 1 h of IMI exposure (162 ± 0.01 % of the control, $p>0.01$). In addition, we observed also a rapid nuclear translocation of activated phosphorylated p38 MAPK after 10 min from IMI stimulation (4000 μM) (Fig. 36C and 37). Nuclear translocation was quantified and was also persistent until 1 h with the highest values of p38 nuclear translocation after 10 min of IMI exposure (2 ± 0.4 % fold increase respect to the control, $p<0.01$).

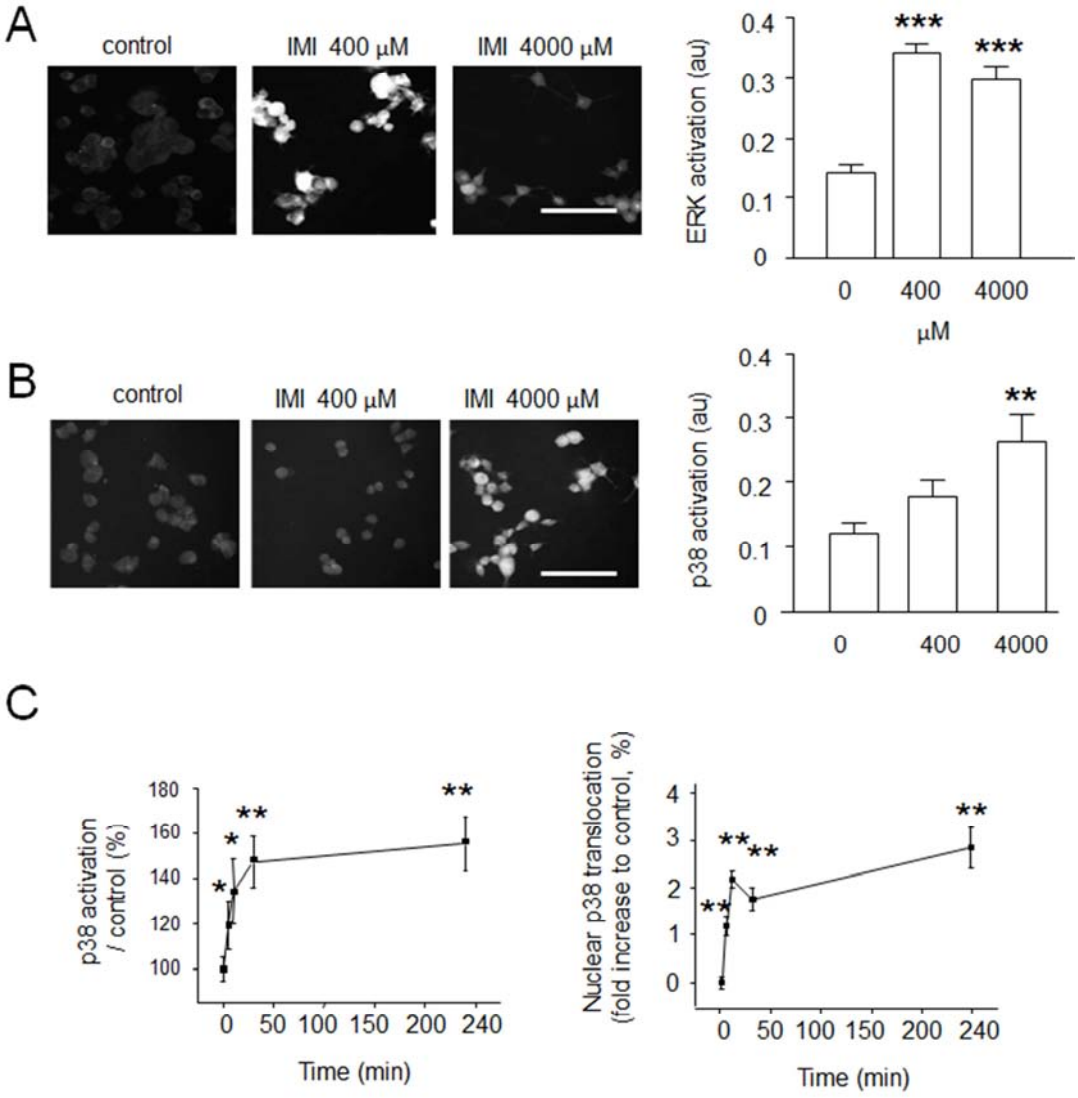


Figure 36: A, B. Representative fluorescence microscopy images of F11 cells in control conditions and in the presence of IMI 400 µM (102.2 mg L^{-1}) and 4000 µM (1022.4 mg L^{-1}) immunostained with antibodies anti-active Erk (A) or active p38 MAPK (B). Scale bar 50 µm. Histograms quantify the grey values of immunopositive signals after background value subtraction. Data have been normalised on total cell number (counterstained with DAPI) and expressed as arbitrary units (AU). To note: different activation profile. C. Graphs quantify p38 signal intensity during the time after application of IMI at 4000 µM to F11 cells (left) and nuclear translocation of activated phosphorylated p38 during time after IMI application (right) (n= 3), $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

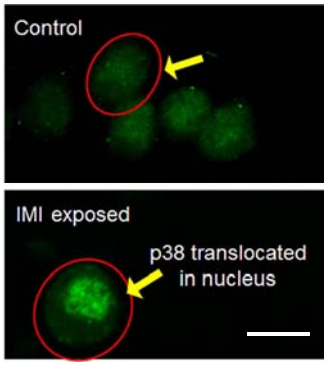


Figure 37: Representative microscopy images of translocated phosphorylated p38 MAPK in nucleus of F11 cells exposed to IMI (4000 µM; 1022.4 mg L^{-1}) for 10 min. Scale bar: 10 µm.

During IMI experiments was explored the activation and response of Nrf2 to IMI. Our analyses indicated that IMI induced a stable and large expression of this transcription factor in a time dependent manner with the highest induction after 30 min of IMI exposure ($p<0.05$) (Fig. 38B). Experiments performed by immunofluorescence, suggested that IMI exposure increased the signal of the transcription Nrf2 factor, proposing the activation of potential rescue secondary responses in F11 cells confirmed by the increase in Nrf2 immunoreactivity (Fig. 38A).

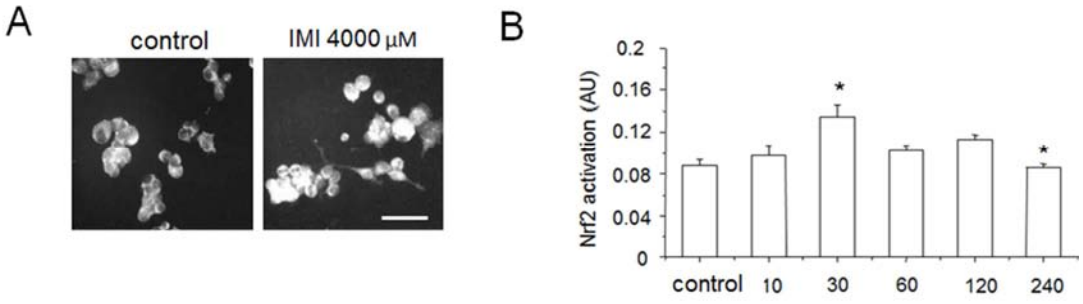


Figure 38: A. Representative example of F11 cells microscopy images analysed in control and after IMI incubation at 4000 µM (1022.4 mg L^{-1}) for 30 min. Cells are immunolabelled with anti-Nrf2 antibodies. Scale bar: 100 µm B. The histogram describes the time course (10, 30, 60, 120 and 240 min) of the relative average quantification of Nrf2 immunoreactivity in control and in IMI-exposed cells (4000 µM; 1022.4 mg L^{-1}). Histograms quantify the grey values of immunopositive signals after background value subtraction. Data have been normalised on total cell number (counterstained with DAPI) and expressed as arbitrary units (AU) (n= 3), $p < 0.05$ (*).

3.1.3. Imidacloprid effects on mitochondrial function and oxidative stress parameters

To further study IMI cytotoxicity, we proceeded with testing of oxidative stress pathways activation. For this purpose, we first performed mitochondria morphological imaging and then we quantified CAT enzymatic activity as well as LP levels to test on-going oxidative stress and possible oxidative damage.

In vivo time course imaging of living cells loaded with JC-1 probe (200 μM; 15 min) and acutely exposed to IMI 4000 μM (1022.4 mg L^{-1}) for 15 and 60 min was performed to quantify IMI-induced mitochondrial stress (Fig. 39A). Qualitative analysis of JC-1 staining indicated a decrease in MMP after IMI treatment. JC-1 dissociate into monomers that emit a green fluorescence pattern as MMP lowers in presence of IMI (4000 μM). This decrease was already significant after 15 min from IMI application with a consequent loss of mitochondrial membrane function (0.7 ± 0.05 fold decrease respect to control; $p<0.05$) (Fig. 39A).

To measure the effects on F11 cells of possible ROS intermediates produced after IMI exposure at 1000 μM (255.6 mg L^{-1}) and 4000 μM for 24 and 48 h, we performed CAT enzymatic assays that revealed an increasing trend of CAT activity in IMI-exposed cells, although not significant ($p>0.05$) (Fig. 39B). TBARS assay performed after IMI exposure at 1000 μM and 4000 μM for 24 and 48 h demonstrated the activation of membrane LP (Fig. 39C) and showed a significant increase in the detection of intermediates of LP in F11 cell medium with respect to control samples. LP values were significantly increased already after 24 h exposure to 1000 μM (6.03 ± 1.2 % fold increase respect to control; $p<0.01$) and to 4000 μM IMI (12.9 ± 4.6 % fold increase respect to control; $p<0.05$). In general, during 24 or 48 h of IMI application, CAT activity in F11 cells showed still a dose-dependent larger increased activation, however not significant ($n =3$, $p>0.05$; Fig. 39B), while significantly higher levels of LP products (TBARS) were found released in cell growth medium exposed to IMI with respect to control ($n=6$, $p<0.05$; Fig. 39C).

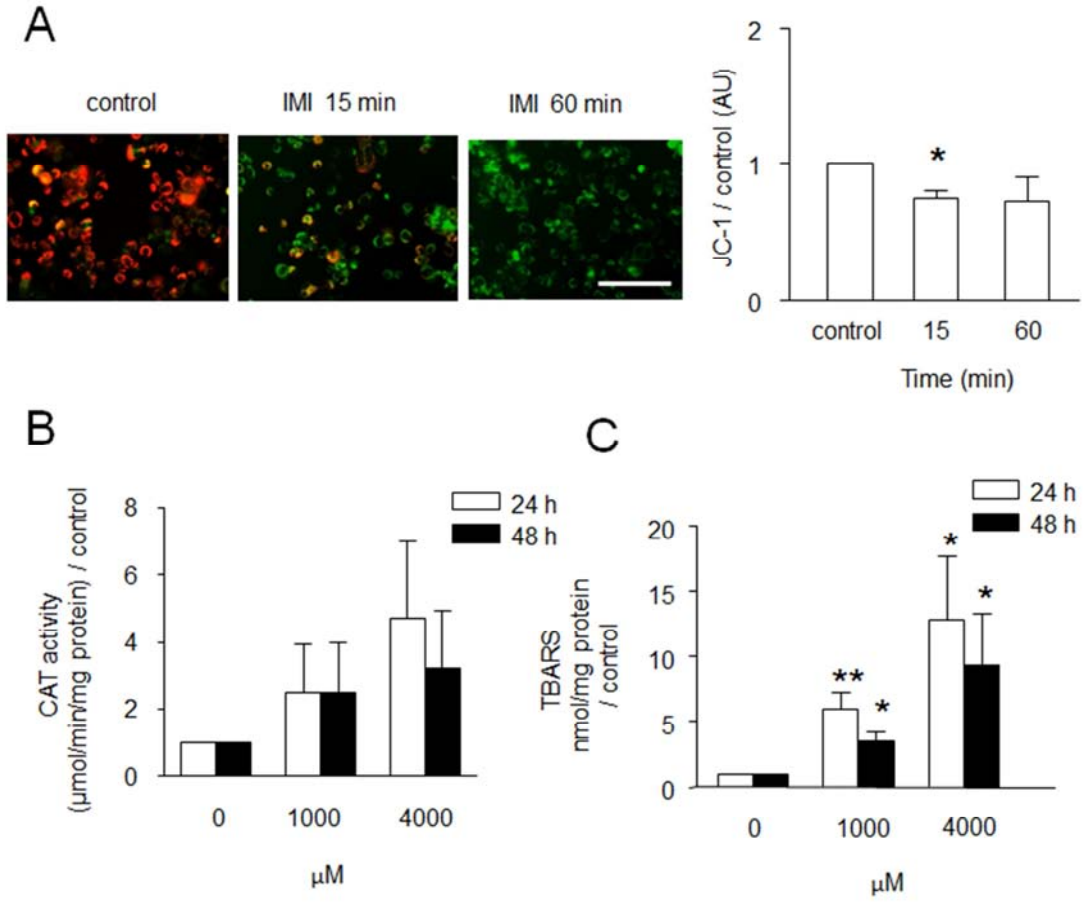


Figure 39: A. In vivo imaging of live cells exposed to IMI 4000 µM (1022.4 mg L^{-1}) 15 and 60 min. Representative microscopy images show JC-1 fluorescence of control (red) and IMI-treated F11 cells (green). Scale bar 100 µm. Histogram represent JC-1 green-red fluorescence signal ratio already significant after 15 min from IMI application. Fluorescence values were expressed as the ratio of fluorescence intensity of J-aggregates (red) to fluorescence intensity of monomers (green); ratio results are presented as fold increase/decrease of the control values (JC-1/control in arbitrary units – AUs) (n=3). B. Catalase activity in F11 cells tested 24 or 48 h after IMI application - 1000 µM (255.6 mg L^{-1}) and 4000 µM (1022.4 mg L^{-1}) (n =3). C. Quantification of TBARS products released in cell growth medium by cell exposed to IMI 1000 µM and 4000 µM for 24 and 48 h (n=3). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

3.1.4. Effects of different tested inhibitors on imidacloprid-mediated cytotoxicity

The role of oxidative stress and p38 induction in IMI cytotoxicity was further confirmed by additional cell survival measurements (Fig. 40A and 40B) performed on cells exposed to 1000 μM (255.6 mg L^{-1}) and 4000 μM (1022.4 mg L^{-1}) IMI for 24 and 48 h cultivated in the presence of *N*-acetyl cysteine (NAC; 1 mM, 30 min pre-application), mixed tocopherols (1 mM, 30 min pre-application) or with the p38 inhibitor SB203580 (10 μM, 30 min pre-application). These inhibitors were tested separately to block possible IMI effects or selected molecular pathways and was noticed that their presence significantly reduced the cell loss. With previous data was demonstrated that MAPK signal transduction pathways as well as IMI-induced oxidative stress and altered oxidative stress parameters are possibly associated to IMI cytotoxic effects. To additionally prove the necessary and sufficient role of these mechanisms, a series of MTT cell viability tests were performed on IMI-exposed cells in the presence of pharmacological inhibitors like p38 MAPK inhibitor SB203580, the broad-range anti-oxidant NAC or LP inhibitor mixture of tocopherols known as vitamin E. These compounds were co-applied with IMI to test their efficacy in protection from IMI endpoint toxic effects after 24 or 48 h incubation (Fig. 40A and 40B).

Oxidative stress inhibitors NAC and vitamin E were sufficient to protect from 1000 μM and 4000 μM (48 h) IMI exposure ($p<0.05$ and $p<0.01$ compared to IMI exposed cells at same time and concentration) (Fig. 40A and 40B). Vitamin E resulted highly effective after 24 h in presence of the highest concentration of IMI (4000 μM) and viable cells reached values of 128 ± 17.6 % ($p<0.05$ compared to IMI exposed cells). A significant protection from 4000 μM IMI toxicity after 24 ($p<0.05$) and 48 h ($p<0.01$) was found also blocking p38 pathways with inhibitor SB203580 (Fig. 40B). Generally, these experiments demonstrated a complex dose- and time-dependent scenario, which demonstrated that oxidative stress inhibitors seem sufficient in some cases to significantly improve the F11 cell survival and to lower the toxic impact of IMI.

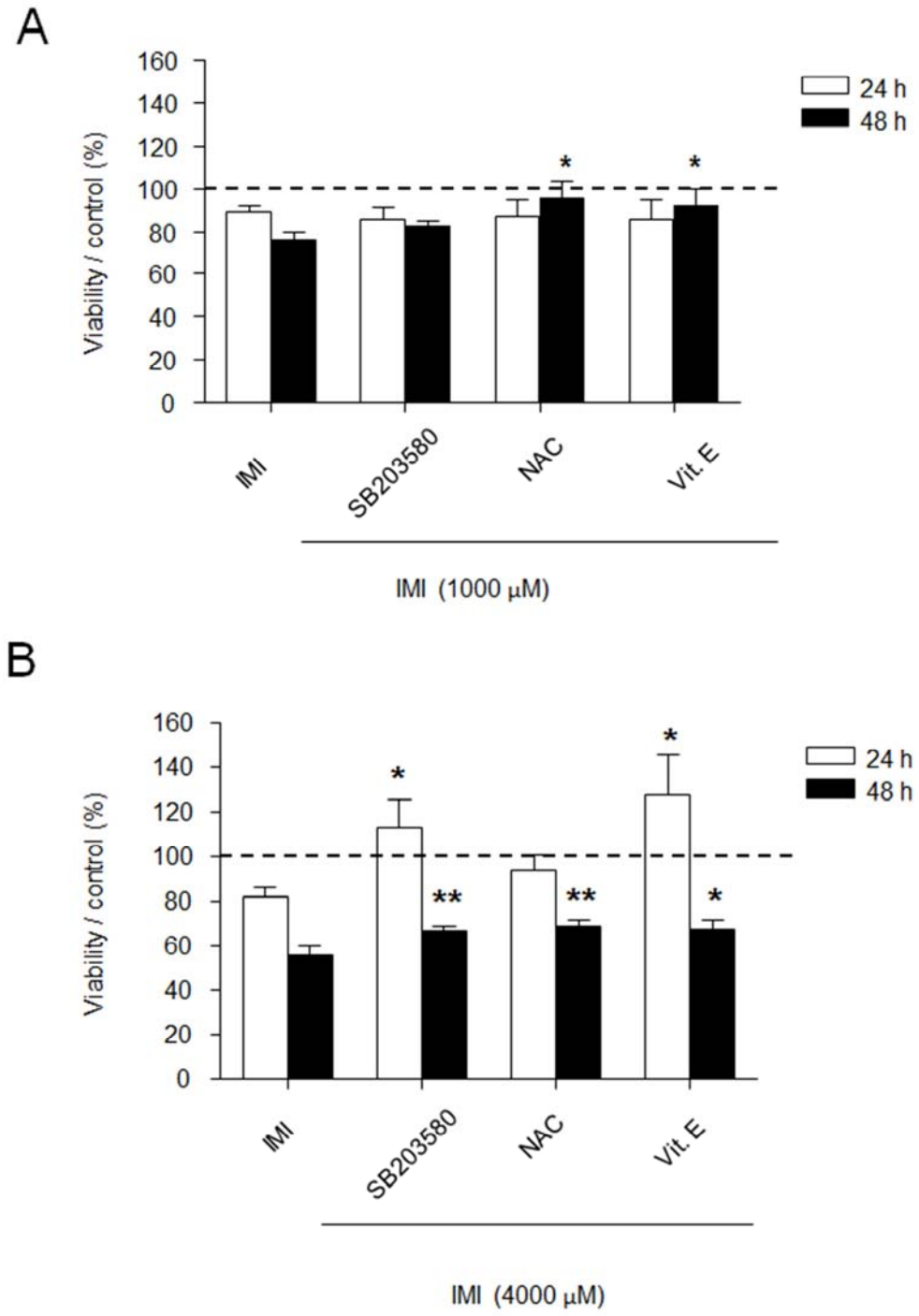


Figure 40: Values of MTT assay in F11 cells after 24 and 48 h exposure to 1000 µM (255.6 mg L^{-1}) (A) and 4000 µM (1022.4 mg L^{-1}) IMI (B) in presence of p38 MAPK inhibitor SB203580, N-acetyl-cysteine (NAC) or mixed tocopherols (vitamin E); data presented as percentage of control shown as dashed line (n=4). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*) compared to IMI-exposed cells.

Toxicity data relative to F11 cells and measured parameters are summarised in Table 12. The lowest concentration of tested chemicals that caused significantly different cell responses from that in the control group was considered as LOEC value of tested compound, while the highest tested concentration at which no significant differences from control were found was the NOEC point. The actual safe or no effect concentration lies between the obtained NOEC and LOEC values for specific biomarker. All obtained data are sorted based on type of response, tested chemical and time of exposure/measurement. Toxicity differed considerably among the tested compounds and among test methods and endpoints. The NOEC and LOEC values for the different chemicals and endpoints ranged from 31.5 to >1022.4 mg L^{-1} (Table 12).

In summary, based on the survival endpoint, the IMI's TP 6CNA clearly results as the most biologically active towards neuronal cells. The toxicity trend (according to cell survival after 48 h) for all tested compound was as follows: 6CNA > Confidor 200SL > IMI > desnitro-IMI > olefin-IMI > 5-hydroxy-IMI. In this toxicity trend is important to notice that desnitro-IMI after 48 h presented significantly higher adverse effect on cell survival compared to IMI (as pure compound or commercial formulation). It reduced the neuronal cell viability of more than 3.3 fold compared to IMI effect alone.

Activation of phosphorylated ERK resulted as the most sensitive endpoint with an early response at IMI concentrations of 400 μM (102.2 mg L^{-1}). Cellular CAT activity was not induced after IMI exposure, but showed an increasing trend. However, elevated cellular ROS production caused consequent peroxidative damage of lipid membrane which was confirmed with elevated values of LP. In general, toxicity tests performed on neuronal cells demonstrated an overall cellular dynamics related to induced oxidative stress with activated Nrf2 factor and MAPK kinases as well as elevated LP.

Table 12: NOEC and LOEC values obtained for applied in vitro models and measured endpoints.

Endpoint	*Chemical* **IMI** *(mg L^{-1})* NOEC	LOEC	**Confidor 200SL** *(a. i. IMI mg L^{-1})* NOEC	LOEC	**6CNA** *(mg L^{-1})* NOEC	LOEC	**desnitro-IMI** *(mg L^{-1})* NOEC	LOEC	**olefin-IMI** *(mg L^{-1})* NOEC	LOEC	**5-hydroxy-IMI** *(mg L^{-1})* NOEC	LOEC
IN VITRO **(F11 cells)**												
Cell survival (24 h)	**25.6**	**51.1**	**255.6**	**1022.4**	**630**	**>630**	**247.1**	**988.5**	**253.6**	**1014.1**	**271.6**	**1086.4**
Cell survival (48 h)	**127.8**	**255.6**	**51.1**	**127.8**	**31.5**	**78.7**	**123.6**	**247.1**	**253.6**	**1014.4**	**271.6**	**1086.4**
ERK induction (30 min)	**<102.2**	**102.2**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
p38 induction (30 min)	**102.2**	**1022.4**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Nrf2 induction (24 h)	**<511.2**	**511.2**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
MMP alterations (15 min)	**<1022.4**	**1022.4**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
CAT (24 h)	**1022.4**	**>1022.4**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
CAT (48 h)	**1022.4**	**>1022.4**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
LP (24 h)	**<255.6**	**255.6**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
LP (48 h)	**<255.6**	**255.6**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*

Abbreviations: n. t. not tested

Table 12: NOEC and LOEC values obtained for applied in vitro models and measured endpoint. (continued)

Endpoint	*Chemical* **IMI** *(mg L^{-1})* NOEC	LOEC	**Confidor 200SL** *(a. i. IMI mg L^{-1})* NOEC	LOEC	**6CNA** *(mg L^{-1})* NOEC	LOEC	**desnitro-IMI** *(mg L^{-1})* NOEC	LOEC	**olefin-IMI** *(mg L^{-1})* NOEC	LOEC	**5-hydroxy-IMI** *(mg L^{-1})* NOEC	LOEC
***IN VITRO* (*S. typhimurium*)**												
Base pair substitution (48 h)	**255.6**	>**255.6**	**255.6**	>**255.6**	**157.5**	**>157.5**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Frame shift mutation (48 h)	**255.6**	**>255.6**	**255.6**	>**255.6**	**157.5**	**>157.5**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*

Abbreviations: n. t. not tested

3.1.5. HPLC and LC-MS analysis of imidacloprid presence in F11 cells

In order to test IMI toxicity on a mammalian cellular model, we tested concurrently the presence of IMI and its TPs in the cell culture medium through HPLC-DAD analyses, while through LC-MS analyses was tested the presence of these compounds inside cells and its capability to enter cellular compartments. These analyses were performed only on the highest concentration of 4000 μM; 1022.4 mg L^{-1} that induced some significant effects on cell survival. High concentration of tested compounds was also selected to ensure possible detection of TPs formed during the incubation period of 48 h. With higher amounts of IMI (up to 1022.4 mg L^{-1}) if the TPs are formed is possible to have them at a detectable/measurable level, otherwise the concentrations were be too low even for highly sensitive methods.

In Fig. 41A are shown representative absorbance traces of HPLC-DAD analysis of cell culture medium containing IMI (4000 μM; 1022.4 mg L^{-1}) as fresh solution (0 h) without cells (blue line), solution of IMI incubated for 48 h without cells (black line) and solution of IMI incubated for 48 h with cells (red line). Incubation of IMI for 48 h at 37 °C reduced the peak signal at 55.7 ± 3.2 % with cells and at 67.8 ± 1.2 % without cells (n=3) compared to control (fresh and not incubated solution of IMI) expressed as C/C_0 [%]. The actual concentration reduction expressed in mg L^{-1} was: for fresh IMI at 0 h (983.4 ± mg L^{-1}), for incubated medium (48 h) containing IMI without cells (685.2 ± 7.7 mg L^{-1}) and with cells (561.8 ± 14.4 mg L^{-1}). These data demonstrated a significant degradation of IMI in absence of cells (after 48 h in dark at 37 °C) and even a stronger IMI disappearance through possible biotransformation processes in presence of cells under the same conditions ($p<0.05$). Due to the applied high concentrations of IMI it was expected the formation and detection of possible TPs. However, at the moment through LC-MS analyses this metabolites released in culture medium were not detected.

In addition, cell lysate were also qualitatively analysed for the presence of IMI and its potential TPs (i.e. metabolites) with LC-MS. Cell lysate chromatograms showed the presence of IMI in the cell lysate (red line), which was confirmed with the extracted ions for the peak at 6.8 min (Fig. 41B and 41C). Also in this case, no emerging of additional peaks was present that could suggest possible biological

transformation of IMI in presence of mammalian cells within 48 h incubation period and these issues are still under investigation.

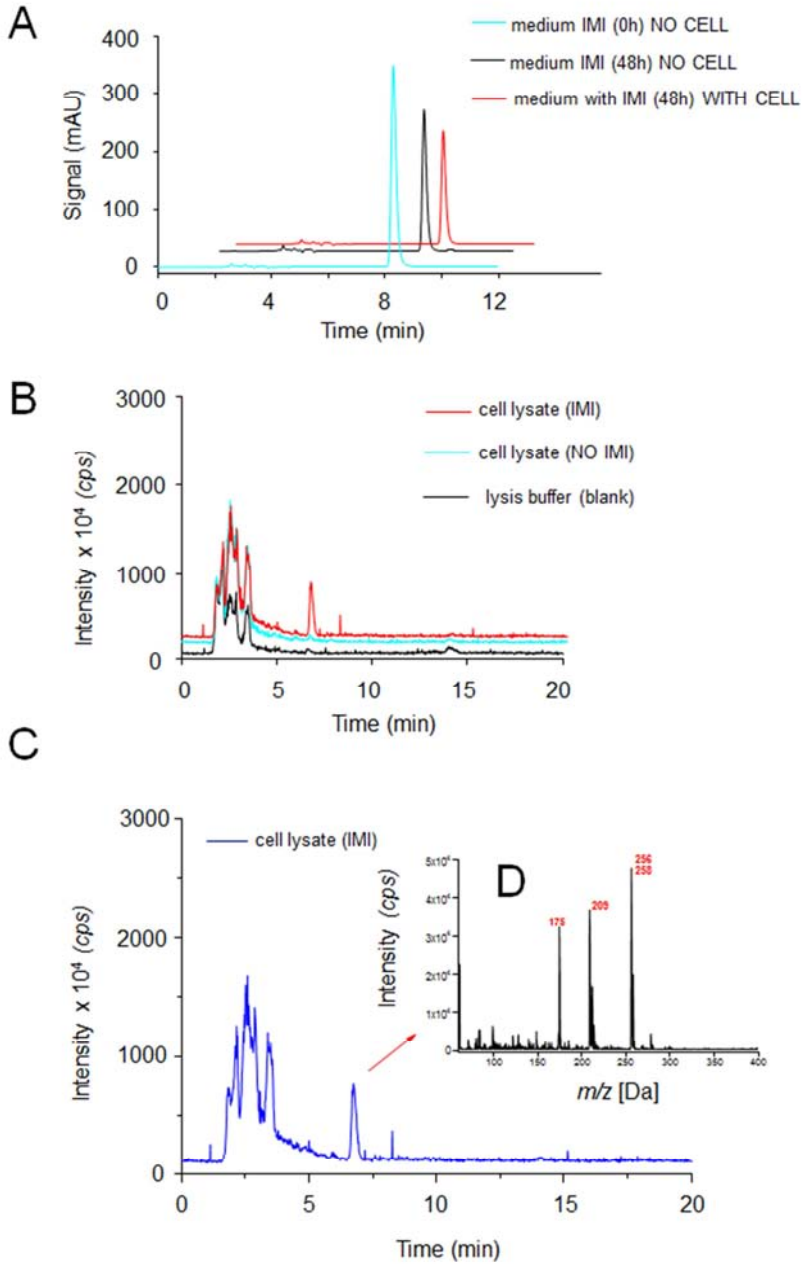


Figure 41: A. Representative peaks of HPLC analysis of cell culture medium samples containing IMI (4000 µM, 1022.4 mg L^{-1}). Fresh IMI (0 h) without cells (blue line), incubated IMI (48 h) without cells (black line) and incubated IMI (48 h) with cells (red line). B. LC-MS total ion chromatograms of cell lysate exposed to IMI (4000 µM; 1022.4 mg L^{-1}) for 48 h (red line) over-imposed to total ion chromatograms of cell lysate in control group without IMI (blue line) and lysis buffer alone as blank (black line). C. Total ion chromatogram of cell lysate treated with IMI with extracted ions for peaks at 6.8 min (1D) (n=3).

3.2. *In vitro* model: *Salmonella typhimurium*

In the *S. typhimurium* reverse mutation assay with metabolic activation IMI tested as pure compound, as Confidor 200SL and its TP 6CNA did not cause any mutations. The determination of mutagenicity induced by IMI and its TP was performed using two bacterial strains (TA98 and TA100) and addition of metabolic S9 fraction in Ames test (ISO 16240, 2005).

The results of the mutagenicity conducted with S9 fraction in *Salmonella* tested strains are presented in Table 13. Aminofluorene (2-AF) and methyl methanesulfonate (MMS) were used as the positive control for mutagen action. The maximum tested doses for each compound was chosen based on non-toxic effect of this dose on bacterial growth. Results revealed that all three tested compounds produced no mutagenicity (at least > or = to two-fold background - control only) colonies with metabolic activation.

It is important to mention that only after Confidor 200SL exposure a minor slight increase of the number of TA100 revertants was noticed, which indicated possible potential for mutagenic impact. The numbers of spontaneous revertants of TA100 is ~100 and for Confidor 200SL were present values of 174 and 183 (but only in two replicates) and were not statistically significant. In summary, no specific mutagenic potential or effect was noticed at the highest tested concentration (Table 12 and 13). These data are in concomitance with previous studies with IMI that did not show any mutations, with or without metabolic activation, in the Ames test (JMPR, 2001).

Table 13: Mutagenicity test responses after treatment with IMI, Confidor 200SL and 6CNA with addition of S9 metabolic fraction in Ames test (n=6).

Chemicals	*His⁺ revertant/plate*[a] Doses – µg/plate (µM)		*Number of revertants* TA98	*Number of revertants* TA100
Positive control (2AF; MMS)	20 µg; 2µL/plate		>2000	>2000
Control (no chemicals)			19.2 ± 1.4	122 ± 2
IMIDACLOPRID				
	0.053	(30)	22.5 ± 1.5	128.5 ± 22.5
	0.178	(100)	25 ± 6	139.5 ± 12.5
	1.78	(1000)	27.5 ± 0.5	132 ± 7
CONFIDOR 200SL				
	0.265	(30)	21 ± 1	150 ± 3.5
	0.89	(100)	26.5 ± 3.5	158 ± 16
	8.9	(1000)	19.5 ± 2.5	161.5 ± 21
6CNA				
	0.033	(30)	26.5 ± 3.5	122.5 ± 6.5
	0.11	(100)	18 ± 1	131.5 ± 4.5
	1.11	(1000)	20.5 ± 0.5	102 ± 1

[a] Data represent the mean revertants ± standard error of data from two independent experiments, each having three replicates (n = 6). The numbers of revertants that are > or = two-fold above that of negative control (without tested chemicals addition) are regarded as the positive response.

3.3. *In vivo* models

In the following sections are presented the results relative to comparative toxicity testing of IMI, Confidor 200SL and four IMI's TP performed on different *in vivo* laboratory bioassay using: *V. fischeri*, *D. subspicatus* and *G. fossarum*.

3.3.1. Toxicity testing with marine luminescent bacteria *Vibrio fischeri*

Toxicity testing of aqueous solutions for three IMI metabolites (5-hydroxy-IMI, desnitro-IMI and olefin-IMI) was performed and the results revealed the highest toxicity in case of olefin-IMI. The toxicity trend for these three compounds was olefin IMI > desnitro IMI > 5-hydroxy IMI. The results are presented in Table 14.

Table 14: Inhibition* V. fischeri *bacterial luminescence after 30 min exposure to 5-hydroxy-IMI, desnitro-IMI and olefin-IMI aqueous solutions.

Concentration chemical [mg L^{-1}]	*Inhibition of luminescence %*		
	olefin-IMI	*desnitro-IMI*	*5-hydroxy-IMI*
0.39	2.25 ± 2.66	4.27 ± 0.58	6.00 ± 0.98
0.78	2.13 ± 0.36	9.74 ± 0.58	7.13 ± 0.37
1.56	6.74 ± 0.18	4.81 ± 0.25	6.53 ± 1.1
3.12	7.06 ± 2.05	4.96 ± 0.58	8.71 ± 0.65
6.25	10.99 ± 2.53	5.76 ± 1	8.88 ± 0.15
12.5	12.5 ± 2.73	9.38 ± 0.05	10.13 ± 0.31
25	20.39 ± 0.67	14.99 ± 0.41	10.73 ± 0.06
50	27.74 ± 1.58	19.79 ± 0	19.54 ± 0.28
100	44.33 ± 0.56	36.6 ± 0.68	34.54 ± 0.09

On the basis of results listed above, the dose response curves for all three TPs were derived. For all three compounds, the inhibition of luminescence with tested concentrations was too low and in this case only EC_{20} values were obtained. The 30 min EC_{20} for olefin-IMI was of 25.08 mg L^{-1}, for desnitro-IMI of 48.23 mg L^{-1} and for 5-hydroxy-IMI of 51.04 mg L^{-1} based on graphical extrapolation from obtained curves (Fig. 42A, B and C). Also, based on obtained Γ values for EC calculation (presented in Annexes), additional graphs with gamma values and its linear regression are shown in Fig. 42a and comparable 30 min EC_{20} values were obtained

(EC_{20} for olefin-IMI was of 22.57 mg L^{-1}, for desnitro-IMI of 41.56 mg L^{-1} and for 5-hydroxy-IMI of 46.01 mg L^{-1}).

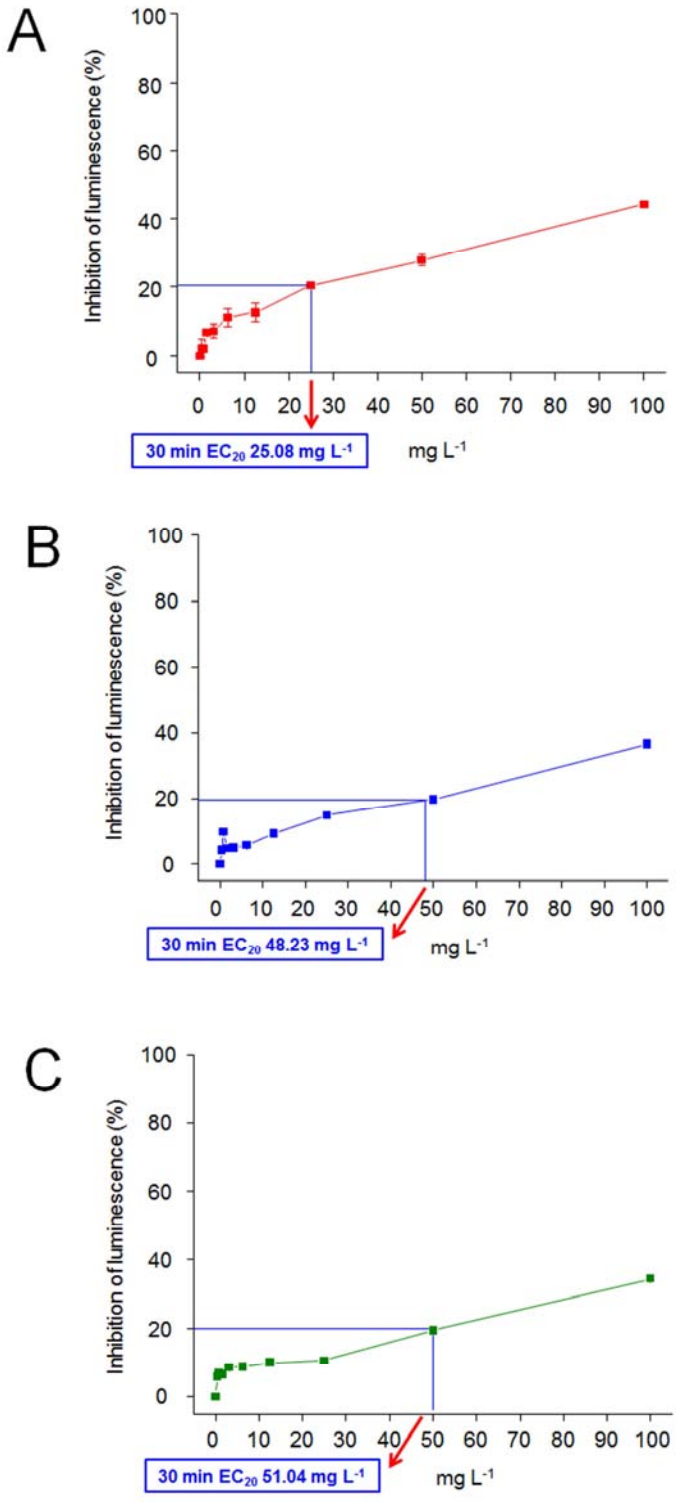


Figure 42: Dose response curve for aqueous solution of olefin-IMI (A), desnitro-IMI (B) and 5-hydroxy-IMI (C) for* V. fischeri *luminescent bacteria within 30 minutes of exposure (n=2).

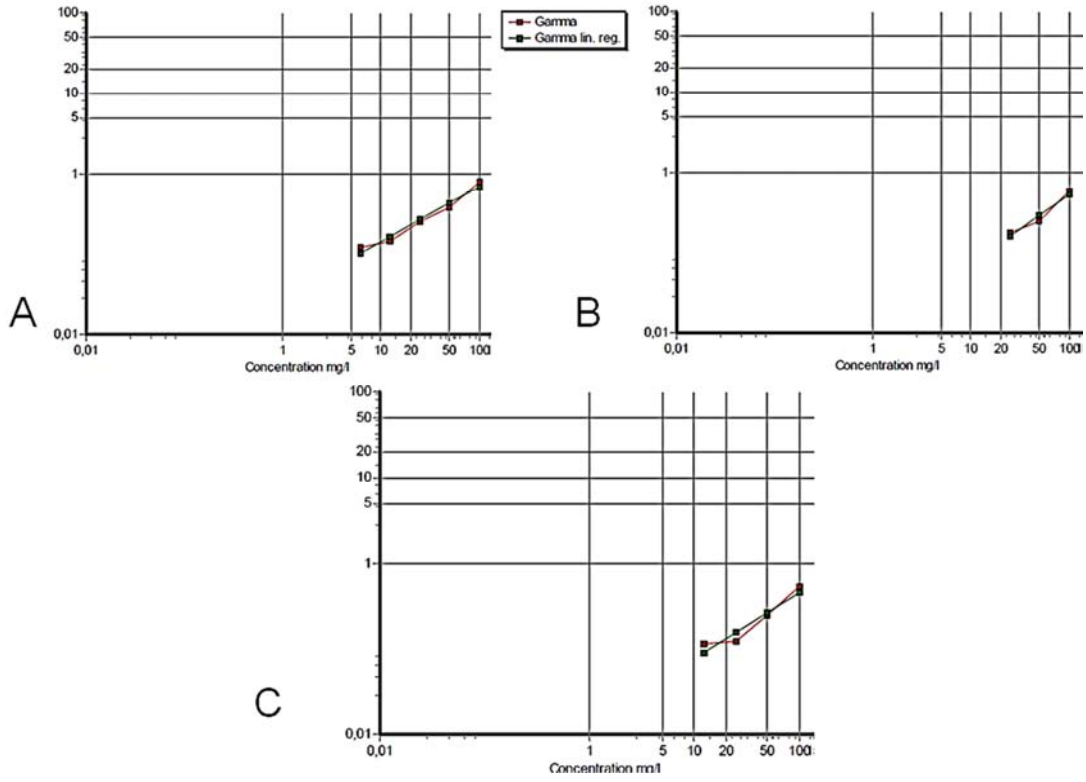


Figure 42a: Gamma values ploted against their corresponding chemical concentration (A) olefin-IMI, (B) desnitro-IMI, (C) 5-hydroxy-IMI. EC_{20} value is given by the point of intersection with the x-axis at Γ = 0.25.

Data on the toxicity of all tested compounds are presented in Table 15 with corresponding EC or IC values. Data relative to IMI, Confidor 200SL and 6CNA are obtained from previous studies and are listed for better comparison of toxic effects within all tested compounds. Based on all EC_{50} and EC_{20} data, 6CNA resulted as the most biologically active and toxic for *V. fischeri* followed by Confidor 200SL and IMI. From three additionally tested IMI's TP, olefin-IMI toxicity could deserve some further consideration and testing.

Table 15: EC and IC values obtained for applied in vivo models and measured endpoints.

Endpoint	*Chemical* **IMI** *(mg L^{-1})*	**Confidor 200SL** *(a. i. IMI mg L^{-1})*	**6CNA** *(mg L^{-1})*	**desnitro-IMI** *(mg L^{-1})*	**olefin-IMI** *(mg L^{-1})*	**5-hydroxy-IMI** *(mg L^{-1})*
***IN VIVO* (*V. fischeri*)**						
Luminescence inhibition (30 min)	**<61.9 IC_{50}=61.9[a]** **IC_{50} =80[b]** **IC_{50}=226[d] (159-322)**	**<56 IC_{50}=56[a]**	**<15.1 EC_{50}=15.1[c]**	**<48.23 EC_{20}=48.23**	**<25.08 EC_{20}=25.08**	**<51.04 EC_{20}=51.04**

[a] Tišler *et al*., 2009

[b] Dell'Arciprete *et al*., 2009

[c] Žabar, 2012

[d] Kungolos *et al.*, 2009

3.3.2. Water quality parameters and stability study with green microalgae and crustacean amphipod

Water quality parameters were measured for all water samples from the sampling site and during toxicity tests with *D. subspicatus* and *G. fossarum*. No significant changes were observed through whole experimental trial (n=10). Mean values were as follows: pH 7.9 ± 0.1, temperature 14.7 ± 0.3 °C and water conductivity of 378.3 ± 21.7 μS cm^{-1}. The water had average oxygen concentration of 9.8 ± 0.2 mg L^{-1} and saturation of 95.8 ± 2.3 %. Mean values of TOC and TN at the water source location were 8.7 ± 0.1 and 0 mg L^{-1}, respectively. Moreover, dissolved oxygen concentration during whole experimental trial was between 70 % and 80 % of saturation. These were all acceptable conditions for toxicity test (EPA, 1996).

Our experiments showed no significant changes in concentration of IMI and 6CNA in test solutions during 24 h (amphipods) and 96 h (algae) exposure (Table 16). The actual exposure concentrations of both chemicals did not differ by more than 3.4 ± 0.3 % (for concentrations in tests with amphipods) and by 15.8 ± 0.4 % (for concentrations in tests with algae) from the initial concentrations. IMI and 6CNA concentrations were consistent over time in all tests (Table 16). Therefore the results are given in nominal concentrations, as suggested by ISO 10706 (2000).

3.3.3. Algae toxicity test

Algal chronic toxicity revealed a high toxic potential of 6CNA at the highest concentration (Fig. 43C). 6CNA induced some perceivable alterations in algae growth, causing slight and temporary inhibition effects at lower doses (4.7 and 15.7 mg L^{-1}) already after 24 h compared to control ($p<0.05$) (Fig. 43C). The highest dose of 6CNA extensively suppressed the algal growth. 6CNA induced acidification of the algal medium (pH up to 5.5 ± 0.1; n=3). In all other groups, pH did not deviate significantly from the initial values as in the case of 6CNA at the highest dose. Overall 6CNA effects were stimulatory on algae growth. Major stimulatory effect of 6CNA was observed at 31.5 mg L^{-1} (48 h) reaching 176.4 ± 3.4 % and stayed significantly increased also after 72 h compared to control ($p < 0.001$) (Fig. 43C). It

was not possible to calculate the IC_{50} value for IMI due to its low inhibitory effects within the entire range of tested concentrations (Fig. 43A). Furthermore, the toxicity of Confidor 200SL ranged from 27.9 % up to 49.72 % (Fig. 43B). Inhibition of algal growth was significant at 127.8 and 255.6 mg L^{-1} compared to control ($p<0.01$). Higher toxicity of Confidor 200SL was possibly induced by known co-formulants present in the commercial formulation which contributed as a major part to toxicity for algae. These co-formulants (DMSO and NMP) alone induced a significant inhibition of 82.3 % and 89.7 % (at 0.06 and 0.12 %; v/v) compared to control ($p<0.001$) (Fig. 43B).

Table 16: Mean ± standard error detected concentrations expressed as µg L^{-1} and mg L^{-1} of IMI and 6CNA in aqueous samples for the 24 h* G. fossarum *and 96 h* D. subspicatus *static toxicity tests (n=3).

Nominal concentration (µg L^{-1})	*G. fossarum* Dark T=22°C [µg L^{-1}]		*Nominal concentration (mg L^{-1})*	*D. subspicatus* Light T=22°C [mg L^{-1}]	
IMI	***0 h***	***24 h***	**IMI**	***0 h***	***96 h***
102.2	105.5 ± 2.5	99.7 ± 0.7	7.6	7.5 ± 0.1	6.4 ± 0.1
153.3	154.7 ± 0.7	148.5 ± 1.4	25.6	26.3 ± 0.5	21.9 ±0.5
204.5	203.9 ± 1.8	198.1 ± 0.5	51.1	51.4 ± 1.2	44 ±1.4
255.6	254.2± 1. 6	250.8 ± 0.4	127.8	127.4 ± 0.7	103.6 ±2.2
511.3	511.7 ± 0.18	481.2 ± 0.6	255.6	255.1 ± 0.8	240.4 ±2.9
6CNA	***0 h***	***24 h***	**6CNA**	***0 h***	***96 h***
62.8	62.4 ± 0.5	63.3 ± 0.9	4.7	4.5 ± 0.1	4.1 ± 0.1
94.6	93.5 ± 0.8	92 ± 0.8	15.7	14.8 ± 0.5	14 ± 0.2
126.2	127.3 ± 0.4	120 ± 0.9	31.5	29.9 ± 0.9	28.7 ± 0.6
157.7	157.4 ± 0.9	152.6 ± 1.1	78.7	77.1 ± 1.3	71.1 ± 0.8
315.5	315.7 ± 0.3	310.1 ± 1.2	157.5	156.1 ± 0.8	122.3 ±2.6

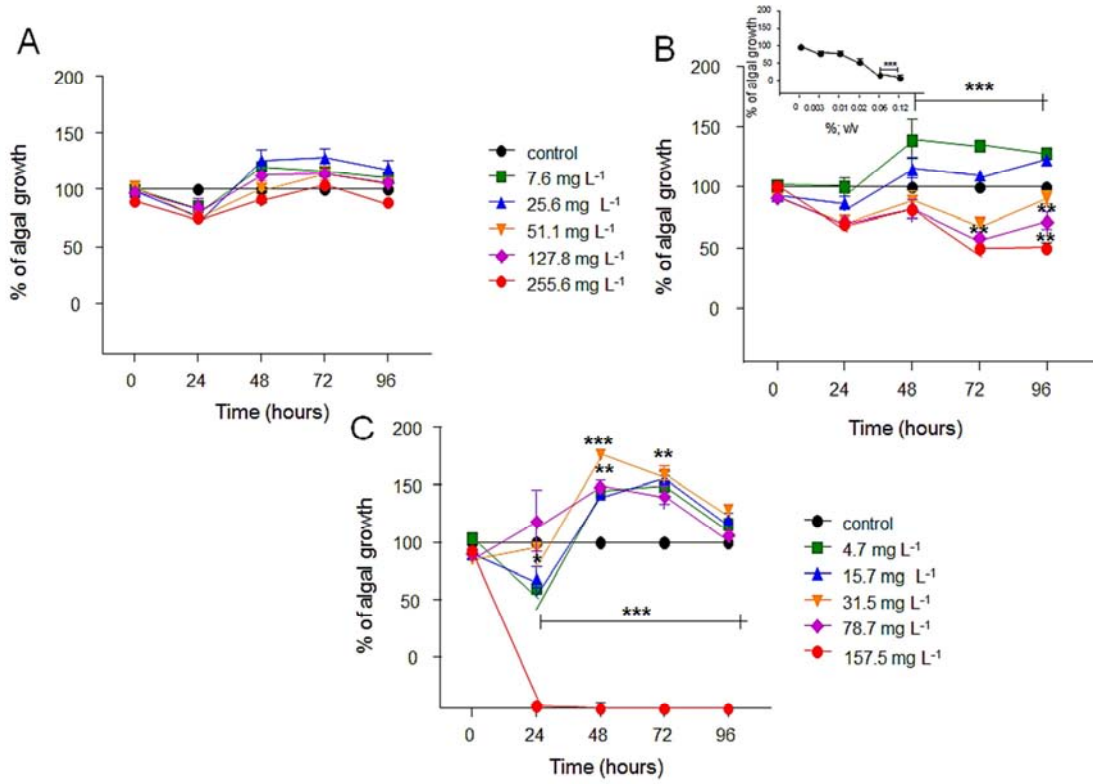


Figure 43: **D. subspicatus** ***% of algal growth compared to control after exposure to IMI (A) Confidor 200SL (B) and 6CNA (C) at 24, 48, 72 and 96 h. The inside graph represents exposure to negative control (known co-formulants only). Data are reported as mean ± standard error (n = 3). p<0.001 (***), p < 0.01 (**), and p < 0.05 (*).***

3.3.4. Amphipods toxicity test

3.3.4.1. Survival rate and behavioural alterations

After 24 h of acute toxicity test, monitored in all groups were: (1) the number of dead amphipods (mortality) and (2) the number of immobile/paralysed or recently moulted amphipods. Only male adult specimens were used for laboratory tests. All specimens presented a mean total body length of 12.35 ± 0.25 mm and mean weight of 0.029 ± 0.002 g. Individuals which sex was not possible to determine were classified as juveniles and not used for this research.

The negative control (mixture of known co-formulants: DMSO and NMP) did not have any adverse effects on *G. fossarum* at all tested concentrations (data present in Annex section). Due to this fact, all values were compared to control (only stream

water). Furthermore, concentrations of all tested compounds lower than 102.2 μg L^{-1} for IMI and 62.8 μg L^{-1} for 6CNA did not induce significant effects compared to control (data present in Annex section).

Average mortality in control groups was between 2.2 ± 1.1 and 4.3 ± 1.9 % in all bioassays. Our data demonstrated slight toxicity of IMI with minor changes in mortality rate (Fig. 44A). IMI induced only 22.3 ± 5.09 % of dead organisms at 102.2 μg L^{-1}. Commercial formulation Confidor 200SL demonstrated an increased effect on mortality, especially at higher concentrations. Percentages of dead organisms at 255.6 and 511.3 μg L^{-1} of a. i. reached 40 ± 5.7 % and 45.5 ± 7.3 %, respectively (Fig. 44A). This increased mortality was significant for the both concentrations ($p<0.05$). On the contrary, 6CNA showed an overall low toxicity, ranging from 8.6 ± 1.9 % up to 14.1 ± 1.1 % (at 62.8 and 315.5 μg L^{-1}, respectively; Fig. 44B).

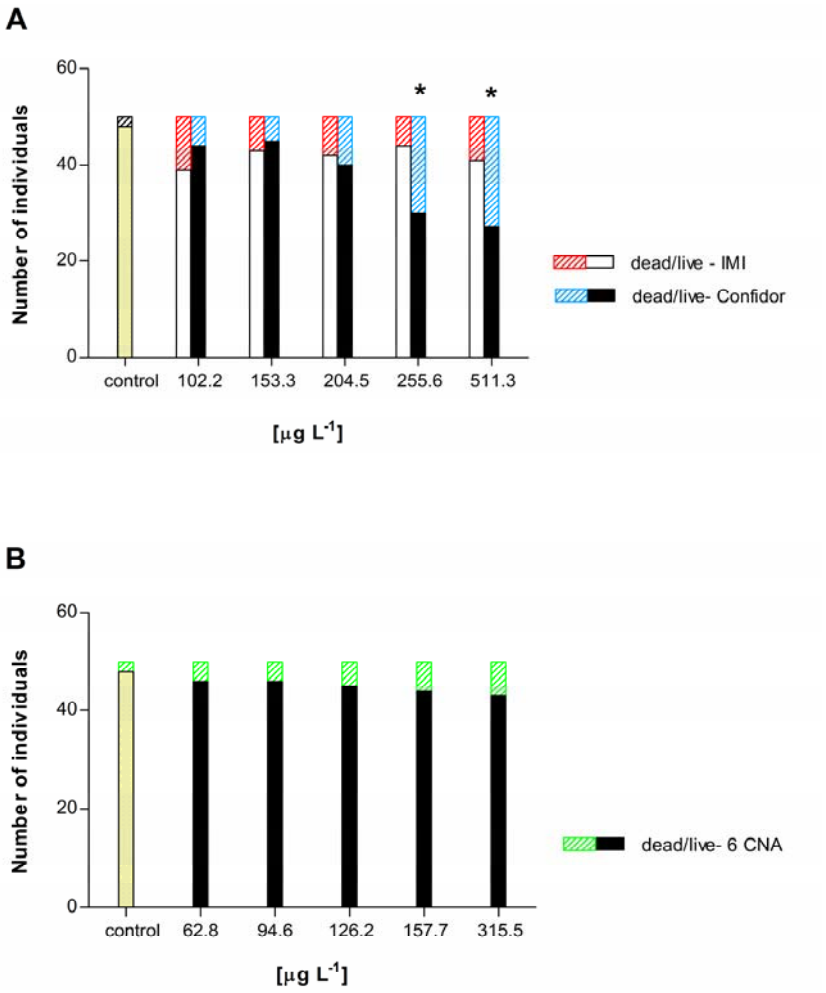


Figure 44: Mortality rate of* G. fossarum *after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). (n=50). p < 0.001 (***), p < 0.01 (**), and p < 0.05 (*).

At 511.3 µg L^{-1} of IMI and Confidor 200SL was present a high number of inactive animals with only respiration movements. These values were of 76.6 ± 6.6 % for IMI and of 90 ± 5.7 % for Confidor 200SL ($p<0.001$; compared to control) (Table 17). It is also interesting to report the number of animals that underwent moult (leaving the entire old *exuvia*) after treatment with tested compounds, apparently stimulatory effect on moult processes was due to the action of transformation product 6CNA. Number of moulted amphipods after 24 h exposure to 6CNA at 315.5 µg L^{-1} was of 56.6 ± 3.3 % ($p<0.001$) (Table 17). Number of moulted animals was minor after 24 h of exposure to IMI and Confidor 200SL at 511.3 µg L^{-1} (23.3 ± 3.3 % and 13.3 ± 3.3 %, respectively; $p>0.05$). 6CNA seemed to induce overall hyperactivity and rapid swimming (with numerous sideways and back-and-forth movements)

which affected 80 ± 5.7 % of total treated gammarids at 315.5 µg L^{-1}. 6CNA (compared to control; $p<0.001$). Numbers of counted individuals which presented the described behavioural characteristic are summarised in Table 17. It is important to emphasise that this data need further quantification with technologies that allow a more detailed analyses and recording of behavioural patterns, also including the fact of high inter-individual variability which make difficulties in data interpretation after statistical analyses (Malev *et al.*, 2012).

Table 17: Percentage of immobile/paralysed, hyperactive and moulted individuals of* G. fossarum *(% of total treated animals) exposed to IMI, Confidor 200SL and 6CNA for 24 h. Data are expressed as mean ± standard error (n=30). p < 0.001 (***), p < 0.01 (**), and p < 0.05 (*).

Nominal concentration (µg L^{-1})	% Immobile/paralysed individuals	% Hyperactive individuals	% Moulted individuals
IMI			
Control	none		none
102.2	16.6 ± 3.3 (**)	None for all groups	10 ± 5.7
153.3	16.6 ± 8.8		13.3 ± 3.3
204.5	13.3 ± 3.3		23.3 ± 8.8
255.6	43.3 ± 3.3 (***)		26.6 ± 3.3 (**)
511.3	76.6 ± 6.6 (***)		23.3 ± 3.3 (**)
Confidor 200SL			
Control	none		none
102.2	23.3 ± 3.3 (**)	None for all groups	6.6 ± 3.3
153.3	33.3 ± 3.3 (**)		13.3 ± 3.3
204.5	46.6 ± 14.5 (**)		13.3 ± 8.8
255.6	56.6 ± 3.3 (***)		10 ± 0
511.3	90 ± 5.7(***)		13.3± 3.33
6CNA			
Control		none	None
62.8	None for all groups	16.6 ± 3.3 (**)	20 ± 5.7 (**)
94.6		23.3 ± 3.3 (**)	33.3 ± 3.3 (**)
126.2		43.3 ± 3.3 (***)	43.3 ± 12 (**)
157.7		43.3 ± 3.3 (***)	46.6 ± 3.3 (***)
315.5		80 ± 5.7 (***)	56.6 ± 3.3 (***)

3.3.4.2 Effects on enzyme activities and lipid peroxidation

Results of enzyme activities were expressed per protein content, since changes in the protein were not significant as a result of 24 h exposure to all tested compounds.

In this study a possible indirect effect of IMI on AChE activity in neonicotinoid exposed gammarids was tested as a biomarker of the cholinergic system. *G. fossarum* exposed to IMI displayed no significant changes of AChE activity at all concentrations (data not presented in graph). The AChE values at all exposure concentrations of IMI ranged between 70.6 ± 7.8 and 78.2 ± 11.6 μmol/min/mg proteins ($p>0.05$; compared to control). CAT activity was not modified after IMI exposure (Fig. 45A). The values ranged between 22.04 ± 1.5 μmol/min/mg protein for control and 28.4 ± 8.6 μmol/min/mg protein at 255.6 μg L^{-1}. Commercial formulation induced a moderate change in CAT at 511.3 μg L^{-1} a. i. going up to 48.06 ± 9.7 μmol/min/mg protein compared to control ($p<0.05$). Values of CAT activity in the case of exposure to 6CNA reached 48.9 ± 6.7 μmol/min/mg protein already at 157.7 μg L^{-1} ($p<0.001$) (Fig. 45B). After exposure to Confidor 200SL two different outcomes for GST activity at 255.6 and 511.3 μg L^{-1} were evident (Fig. 46A). At 255.6 μg L^{-1} was present an observable, but statistically not significant decrease in GST activity ($p = 0.053$). The values of GST went from control values of 419.1 ± 101.8 nmol/min/mg protein to 286.8 ± 92.71 nmol/min/mg protein at 255.6 μg L^{-1}. Higher concentration of Confidor 200SL (511.3 μg L^{-1} of a. i.) induced an increase of GST activity up to 831.4 ± 117.2 nmol/min/mg protein ($p<0.05$). IMI and 6CNA exposure provoked no significant changes in GST activity compared to control ($p>0.05$) (Fig. 46A and 46B, respectively). IMI induced at 102.2 μg L^{-1} an increase in LP levels (Fig. 47A). This increase was 2.7-fold higher in contrast to the control group ($p<0.01$). On contrary, Confidor 200SL induced significant rise of TBARS only at higher dose (255.6 μg L^{-1} of a. i.; $p<0.05$). This increase was lower than the significant peak induced by IMI at 102.2 μg L^{-1} (Fig. 47A). No significant effect of 6CNA on LP increase was noted after 24 h at all concentrations (Fig. 47B). However, it was detected a significant decrease of LP values at 315.5 μg L^{-1} compared to control ($p<0.001$).

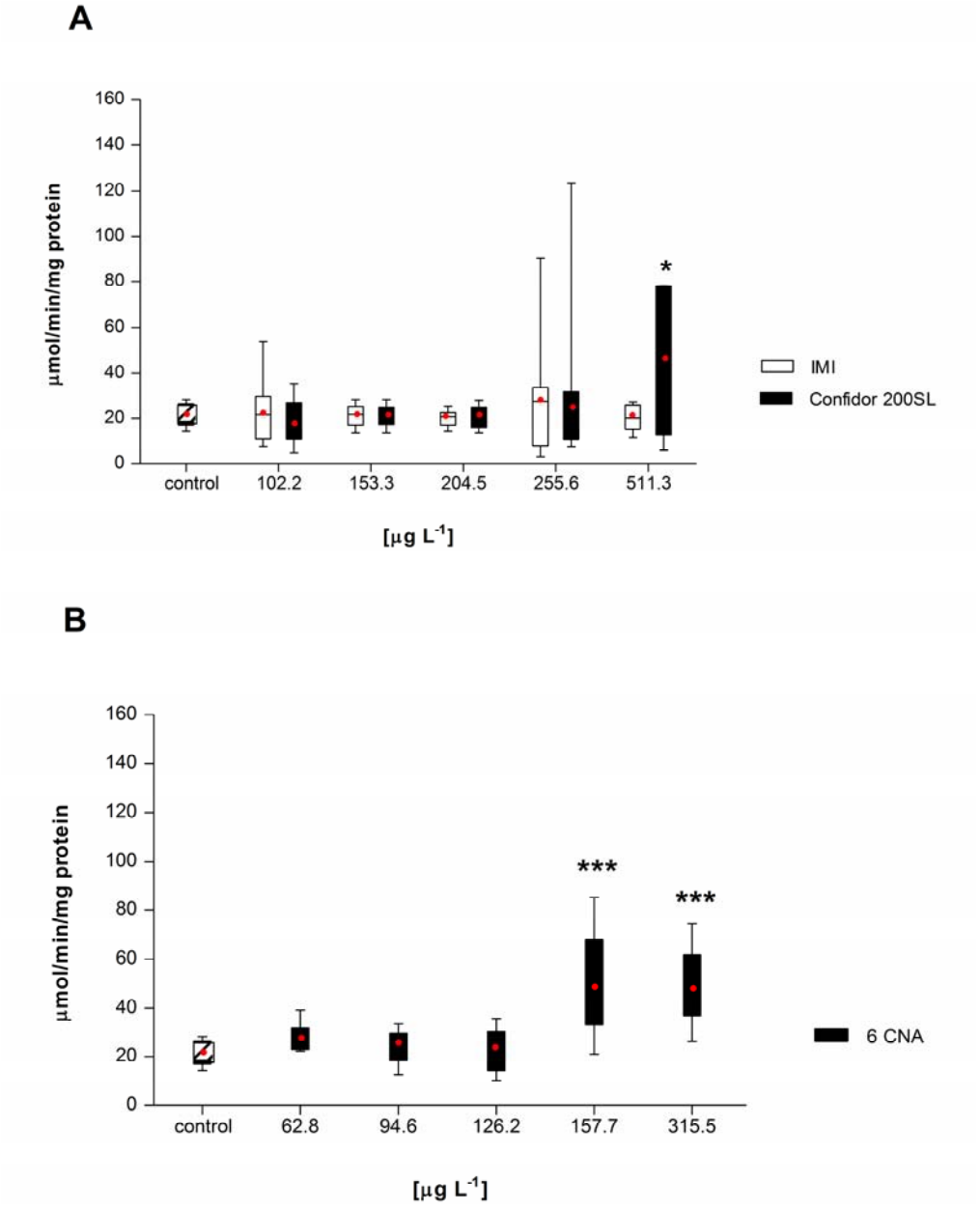


Figure 45: Whole-body CAT activity (µmol/min/mg protein) of* G. fossarum *measured after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75 % of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). (n=10). p < 0.001 (***), p < 0.01 (**), and p < 0.05 (*).

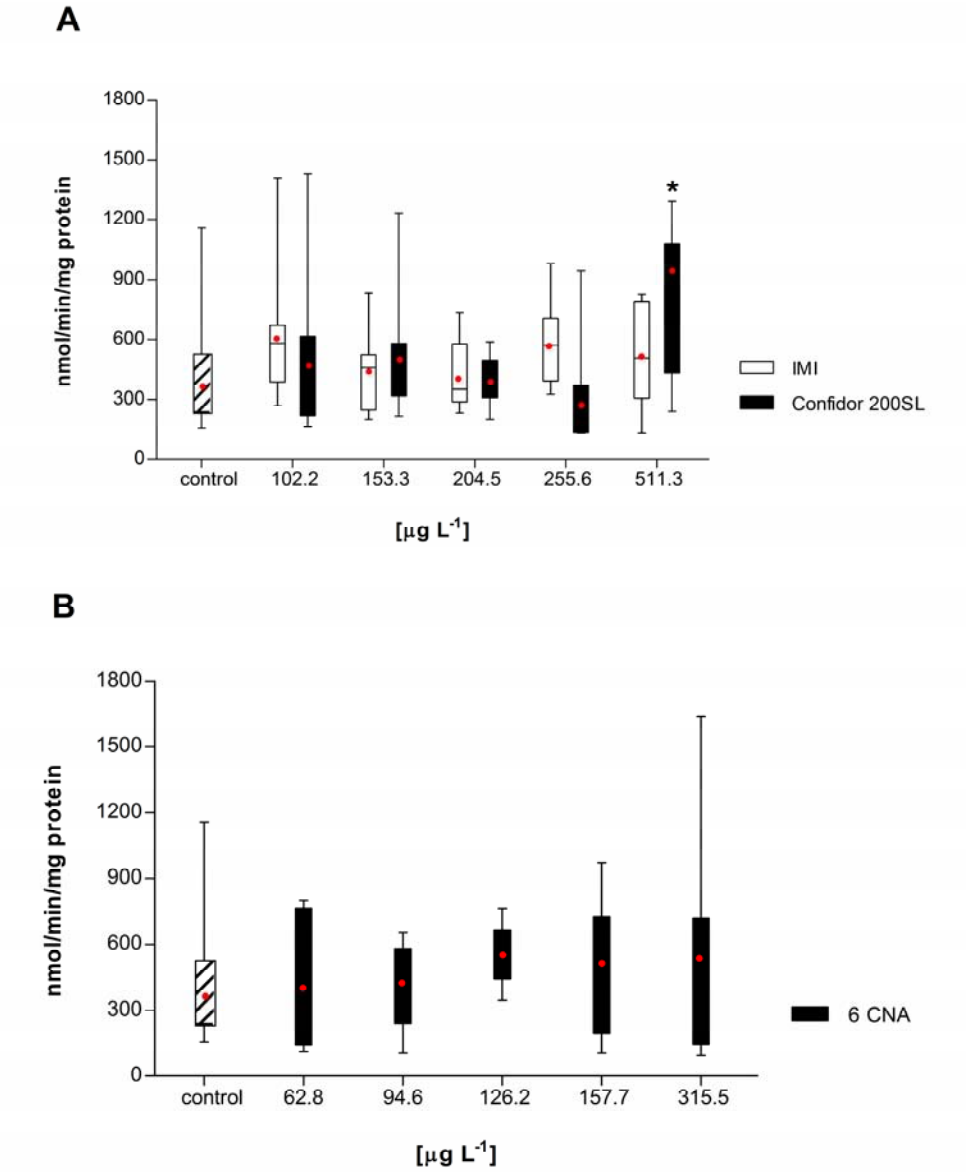


Figure 46: Whole-body GST activity (nmol/min/mg protein) of* G. fossarum *measured after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75 % of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). (n=10). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

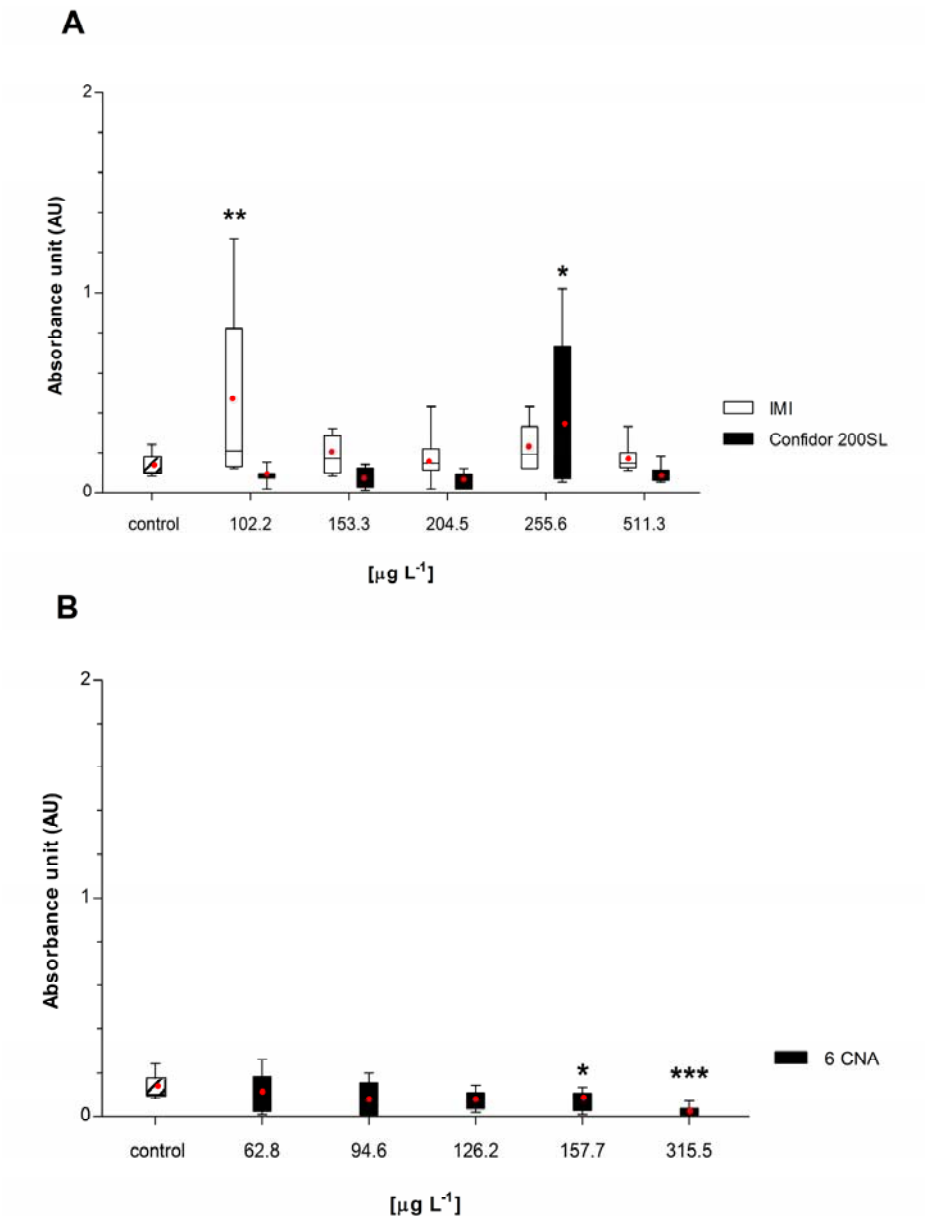


Figure 47: Whole-body LP of* G. fossarum *(expressed in absorbance units of TBARS products) measured after 24 h of exposure IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75 % of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). (n=10). p < 0.001 (***), p < 0.01 (**), and p < 0.05 (*).

3.3.4.3 Effects on MXR mechanism

Contaminants in ecosystems can occur as complex mixture, so it is important to consider their possible synergistic, additive or antagonistic action and to understand the interactions between stressor to identify the appropriate application of laboratory studies to the environment (Herbrandson *et al*., 2003). To test possible synergistic effect was selected copper sulphate ($CuSO_4$) which can be found as a solution in most running waters. $CuSO_4$ is in large amounts used as fungicide in agriculture and could interact with neonicotinoid insecticide (i. e. IMI).

The testing of MXR potential in *G. fossarum* was performed by the accumulation version of the exclusion dye assay (using Rh B as a model MXR substrate). Obtained data are shown in Fig. 48. The animals were exposed to IMI or Confidor 200SL (3 or 30 μM; 0.7 or 7.6 mg L^{-1}) and 6CNA (3 or 30 μM; 0.4 or 4.7 mg L^{-1}) and copper sulphate (0.75; 3 and 10 μg L^{-1} copper a.i.), as pure compounds and mixtures.

In the presence of high concentrations of MXR inhibitors (20 μM VER and 10 μM CA, respectively) the accumulation of Rh B in the whole body of amphipods increase of 112.3 ± 30.1 % (VER) and 78.4 ± 22.2 % (CA) in comparison to control (100 ± 0.0 %). VER resulted as a better competitive inhibitor, which contends with active sites for the dye and the animals were more sensitive to its presence.

No inhibitory effect of IMI and its commercial formulation was observed vs. control. Interestingly, lower concentration of 6CNA had inhibitory effect on membrane transporters causing accumulation of Rh B (205.2 ± 49.9 %; $p<0.05$).

Rh B accumulation in whole body exposed to mixture of IMI and copper (10 μg L^{-1}) as mixture was of 194.3 ± 82.8 %, while for amphipods exposed to 6CNA and copper (10 μg L^{-1}) was of 196.4 ± 17.4 % higher in comparison to control ($p<0.05$). This inhibitory effect was relevant and significant showing dye accumulation and possible synergistic effect of IMI with copper in *G. fossarum.* It is important to note high inhibitory effect of 6CNA alone which exclude possible synergism with copper as in case of IMI. (Fig. 48).

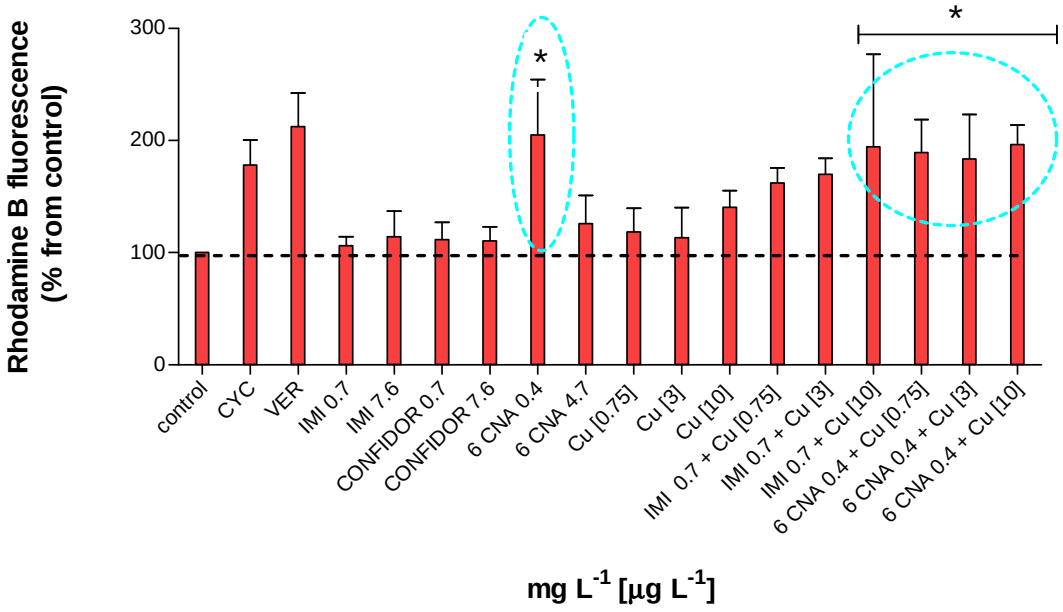


Figure 48: Inhibitory potential of IMI, Confidor 200SL and 6CNA (0.7 or 7.6 mg L^{-1}) and copper (3 and 10 µg L^{-1}) on the accumulation of fluorescent Rh B dye in* G. fossarum*. Results are expressed in fluorescence units (f.u.) normalised on total animal wet body weight expressed as percentage average of n=3 separated experiments. $p < 0.05$ (*).

IMI, Confidor 200SL and 6CNA toxicity to *D. subspicatus* and *G. fossarum* in 24 h (acute) and 96 h (sub-chronic) exposure was also expressed as NOEC and LOEC values based on measured parameters – endpoints (Table 15).

It is clearly shown a different effect mode of action of IMI/Confidor 200SL and 6CNA on freshwater algae growth. IMI tested alone as pure compound showed no significant effects on algae, while Confidor 200SL, due to known co-formulants present in the marketed mixture induced significant adverse and inhibitory effect on algal growth. On contrary, 6CNA had a stimulatory and hormetic effect that induced strong algal proliferation at all concentrations ≤ 78.7 mg L^{-1}. These specific effects and highly diverse algae responses after exposure to IMI and its TPs confirmed measurement of algae growth as a suitable biomarker for neonicotinoid toxicity testing.

In amphipods was confirmed also a different toxic mechanism of IMI/Confidor 200SL and 6CNA. In specific this effect was noticed on level of physiological biomarker such as behavioural alterations. IMI and Confidor 200SL

induced mostly amphipod immobilisation, while 6CNA was affecting through amphipod hyperactivity and increased moult frequency. Based on high-level (physiological) measured endpoint such as behaviour (24 h) the toxicity trend of tested compound could be presented as: Confidor 200SL< IMI < 6CNA, while based on mortality this trend was slightly modified: IMI < 6CNA < Confidor 200SL.

The NOEC and LOEC values for the different chemicals and endpoints in amphipods ranged from 62.8 to 7600 μg L^{-1} of all tested chemicals (Table 15 continued). In general, was shown a complex reaction to the tested chemicals (i.e. 6CNA) with specific behavioural response and induced oxidative stress. In addition, were noticed possible effect on membrane level; peroxidative damage of lipid membrane as well as effect on level of membrane transporters (e.g. MXR endpoint). This latter biomarker results as a useful early signal for fast screening short-term (1 h) acute neonicotinoid exposure at higher concentrations, which needs further considerations and standardisation.

Table 15a: NOEC and LOEC values obtained for in applied vivo models and measured endpoints. (continued)

Endpoint	*Chemical* **IMI** *(mg L^{-1})* NOEC	LOEC	**Confidor 200SL** *(a. i. IMI mg L^{-1})* NOEC	LOEC	**6CNA** *(in mg L^{-1})* NOEC	LOEC	**desnitro-IMI** *(mg L^{-1})* NOEC	LOEC	**olefin-IMI** *(mg L^{-1})* NOEC	LOEC	**5-hydroxy-IMI** *(mg L^{-1})* NOEC	LOEC
***IN VIVO* (*D. subspicatus*)**												
Algae growth inhibition (24 h)	**255.6**	**>255.6**	**<255.6**	**255.6**	**<4.7**	**4.7**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t*
Algae growth proliferation (24 h)	*n. e.*	*n. e.*	*n. e.*	*n. e.*	*n. e.*	*n. e.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Algae growth inhibition (48 h)	**255.6**	**>255.6**	**<25.6**	**25.6**	**<157.5**	**157.5**	*n. t.*	*n. t*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Algae growth proliferation (48 h)	**<25.6**	**25.6**	**<7.6**	**7.6**	**15.7**	**31.5**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Algae growth inhibition (72 h)	**255.6**	**>255.6**	**51.1**	**127.8**	**<157.5**	**157.5**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Algae growth proliferation (72 h)	**<25.6**	**25.6**	**<7.6**	**7.6**	**15.7**	**31.5**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Algae growth inhibition (96 h)	**255.6**	**>255.6**	**51.1**	**127.8**	**<157.5**	**157.5**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Algae growth proliferation (96 h)	**255.6**	**>255.6**	**<7.6**	**7.6**	**<31.5**	**31.5**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*

Table 15a: NOEC and LOEC values obtained for applied in vivo models and measured endpoints. (continued)

Endpoint	*Chemical* **IMI** *(in µg L^{-1})* NOEC	LOEC	**Confidor 200SL** *(a. i. IMI in µg L^{-1})* NOEC	LOEC	**6CNA** *(in µg L^{-1})* NOEC	LOEC	**desnitro-IMI** *(in µg L^{-1})* NOEC	LOEC	**olefin-IMI** *(in µg L^{-1})* NOEC	LOEC	**5-hydroxy-IMI** *(in µg L^{-1})* NOEC	LOEC
IN VIVO (*G. fossarum*)												
Mortality (24 h)	**511.3**	*n. e.*	**204.5**	**255.6**	**315.5**	*n. e.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
Behavioural alterations (24 h)	**<102.2**	**102.2 (immobile)**	**<102.2**	**102.2 (immobile)**	**<62.8**	**62.8 (hyperactive)**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
	204.5	**255.6 (moulted)**			**<62.8**	**62.8 (moulted)**						
AChE (24 h)	**511.3**	*n. e.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
CAT (24 h)	**511.3**	*n. e.*	**255.6**	**511.3**	**126.2**	**157.7**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
GST (24 h)	**511.3**	*n. e.*	**255.6**	**511.3**	**315.5**	*n. e.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
LP (24 h)	**<102.2**	**102.2**	**204.5**	**255.6**	**126.2**	**157.5 (decreased LP)**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*
MXR (1 h)	**7.6 mg L^{-1}**	*n. e.*	**7.6 mg L^{-1}**	*n. e.*	**<0.4 mg L^{-1}**	**0.4 mg L^{-1}**	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*	*n. t.*

Abbreviations: n. e. not evaluated; n. t. not tested

DISCUSSION

4. Discussion

In vitro

The utilisation of *in vitro* test models such as immortalised neuronal cell lines is growing of importance in human and environmental toxicology. All interactions between chemicals and biota occur firstly on the level of cell. Therefore, the use of cell culture in assessment and detection of possible toxic effects of chemical (e.g. pesticides) can be useful (Segner and Braunbeck, 1998). Great amounts of different pesticides, especially insecticides are used worldwide and 5 % of whole population (mainly agricultural workers) are directly exposed to them. According to recent reports, this part of population is calculated to be of 2.6 million persons (David, 2004; Najafi *et al*., 2010). Chronic exposure to pesticides has been associated in humans to several diseases of nervous system, including neurodegenerative diseases and peripheral neuropathies in adult and childhood (Eskenazi *et al*., 2008). Prolonged pesticides affecting of neurotransmitter release or receptors operation in human nervous system may lead to synaptic plasticity, long-lasting hypersensitisation or induction/attenuation of molecular mechanisms for neuronal cell survival.

All these effects present a novel area of research which needs further clarifications because there are still a limited number of studies that are referring to IMI in terms of both contaminant and as potential hazard for the additive/cumulative effects on human health. Recently, research in this field is concentrating on IMI and its TPs-mediated toxic effects with biochemical and molecular approaches performed on suitable neuronal cell lines (Table 5 and 6). When dealing with the issue of IMI effects on signalling pathways one should mention the well-known fact that both inorganic and organic chemicals can stimulate ROS production (Viarengo *et al*., 2002).

IMI and its TPs also elicit acute intracellular responses, particularly in relation to signal integration pathways in mammalian cells. In neuroblastoma cells, low levels of these compounds (i.e. IMI and desnitro-IMI) activate the extracellular-regulated MAPK kinase cascade (in specific ERK) via nAChRs and intracellular calcium mobilisation, leading to possible attenuation of neuronal functions (Tomizawa and Casida, 2002). In our study, F11 cells treated with IMI showed short-

term and transient p38 and ERK MAPK activation, while inhibition of p38 MAPK, blocked and attenuated IMI-induced cell death proving its involvement in oxidative stress pathway. Pesticides are known to induce oxidative stress by generation of free radicals that might cause LP, alternations in membrane fluidity and DNA damage. El-Gendy *et al.* (2010) observed a marked increase in the activities of the antioxidant enzymes SOD and CAT after IMI treatments in adult mice. Since these enzymes are the first lines of defence against the oxygen free radicals, such increase in the activities has been attributed to the defence mechanisms against oxidative stress in the process of attempted cellular repair. In the same study, IMI treatment to mice indicated a marked increase in the hepatic LP. These results are parallel to the data of Khan and Kour (2007); Yu *et al.* (2008); Duzguner and Erdogan (2010) and Kapoor *et al.* (2011) with increased LP values, altered SOD or CAT activity and decrease in GSH content suggesting that induction of oxidative stress is perhaps the central way by which IMI exerts its cytotoxic effects (Khan and Kour, 2007; El-Gendy *et al*., 2010). Our data demonstrated a moderate toxicity of IMI, however associated with activation of stress oxidative responses. A possible accumulation of intracellular ROS induced by IMI-exposure led to increased levels of LP and slightly improved CAT activity. These results indicate that the increase in oxidative stress has a potential crucial role in the neurotoxicity of IMI in F11 cells.

Oxidative stress is one of the most important pathways leading to neuronal cell death (Lee *et al*., 2011). This phenomenon is commonly observed when free radicals, such as ROS or RNS overcome cellular antioxidant system and induce cellular damage. ROSs and subsequent oxidative damage lead to disruption of cellular membrane integrity and mitochondrial dysfunctions. In F11 cells, IMI (at higher concentration) affected also mitochondria causing substantial decrease of MMP and consequent loss of mitochondrial activity. In this study was observed that incubation of F11 cell with IMI induced oxidative stress and that IMI-induced cell death was diminished by pre-treatment with antioxidants (i.e. NAC known as vitamin C and vitamin E). NAC can directly and rapidly scavenge free radicals or quench their reactivity and can act by up-regulating endogenous antioxidant defences. It also, fights off several pollutants by stimulating enzymes that detoxify the cell/organism (El-Gendy *et al.*, 2010). Vitamin E is also a naturally occurring lipid soluble antioxidant which protects cells against membrane LP and expression of peroxidation

products including MDA (Jaine *et al*., 2000). Pre-treatment with antioxidant compounds decreases the generation of ROS preventing the pesticide instability in the antioxidant enzyme activities (Niki *et al.*, 1995; El-Gendy *et al.*, 2010). Our results have revealed that the pre-treatment of neurons with NAC and vitamin E detoxifies IMI-induced neuronal toxicity due to protection of cells against IMI-induced toxic effects (inhibition of mitochondrial activity, LP and ROS action). Presented data are in accordance with those of Verma *et al.* (2007) and El-Gendy *et al.* (2010) who confirmed that pre-treatment with vitamins E and C protected rats from chlorpyrifos-induced oxidative stress and diminished its toxic effects.

Interestingly, the IMI-dependent effects were different in the F11 serum growing line with respect to starved neurons (differentiated), suggesting a different molecular action *via* specific elements in the cell, presumably related to a mature neuronal phenotype. Starved neurons were more susceptible to IMI effect with 50 % of cell loss already at 340 µM (86.7 mg L^{-1}) (still under investigation). A detailed functional study of the effect of IMI on F11 sensitisation is yet to be explored and may be achieved by functional studies like calcium imaging or patch clamp on IMI-treated cells. A relevant morphological difference was evident between control cells and those exposed to high dose of IMI. These differences were observed as an important change in the monolayer cell density when compared to control as opposed to distinguished morphological changes and damage, which were also visible. Despite the subjectivity involved identifying morphological alterations, all fluorescence/light microscopy analyses and methods applied in this research were definitely a useful helper in verifying the result of the cytotoxicity test (Holler *et al*., 2000).

The F11 cellular model used in this study was sensitive to IMI activation and showed consequent signs of stress. The cytotoxicity of IMI appeared to be concentration-dependent in the cell viability test using an MTT assay. IMI resulted as moderately toxic to F11 cells with a cell loss of ~ 50 % only at concentrations higher than 4000 µM (1022.4 mg L^{-1}) after 48 h of exposure. In case of Confidor 200SL this same effect after 48 h was observed at concentrations lower than 4000 µM a. i. IMI. No significant effect on cell survival were observed at concentrations lower than 200 µM (51.1. mg L^{-1}) for IMI and 500 µM (127.8 mg L^{-1}) for Confidor 200SL. In a study of Skandrani *et al*. (2006) with differentiated dopaminergic neuronal cell line

SH-SY5Y, 50 % of cell death was reached after 72 h exposure at 2.5-fold lower concentration of IMI (1565 μM; 400 μg mL^{-1}) and 5-fold lower concentrations of Confidor 200SL (782 μM; 200 μg mL^{-1}). Even if in both studies, cytotoxicity was related to relatively high tested concentration, it is evident that commercial formulations were more aggressive to biological models than the pure compound, when compared to similar concentrations of active principle. Similar observations were reported for marketed mixtures of various pyrethroids and carbamates (Bonatti *et al*., 1994; Skandrani *et al*., 2006). Information on inert ingredients is largely treated as business secret, but these chemicals have been shown to exert additive or synergistic toxicity, due to their specific mode of action or by increasing the bioavailability and effectiveness of the a. i. (Beggle *et al*., 2010). Co-formulants and additives used in pesticide formulation may present themselves as very active, biologically or chemically, and can be also toxic. At the moment more than 500 additives are used as active ingredients (Cox and Surgan, 2006; De Silva *et al.*, 2010). Moreover, it is rare for the population to be exposed to insecticides as pure ingredients and the increase in toxicity of formulation raises more concerns and should be further studied.

It is still unclear if, the IMI evoked cytotoxicity, is a direct or indirect effect. One possibility is that IMI, acting as agonist of nAChRs (Tomizawa and Casida, 2003) causes a persistent neuronal depolarisation and as a consequence it may induce the release of molecules or peptides (ATP, glutamate or CGRP) with associated toxic effects as well as characteristic features of mitochondrial intrinsic apoptotic pathway such as activation of caspase-9 and -3 and nuclear condensation observed by Hoescht (DAPI) staining in our study. However, pre-incubation with a specific antagonist of nAChR α3 subunit which are homogenously highly expressed in F11 cells (mecamylamine; MEC, Arias *et al.* 2010) prevented only partially IMI cytotoxicity, suggesting more complex effects that originate also in a nAChR-independent manner and that is impossible to exclude multiple molecular targets of IMI.

Besides IMI are relevant also IMI's TPs (metabolites) which have been discussed in other sections of this manuscript. Since all of the *in vivo* toxicology studies on IMI involve the generation of TPs (i. e. metabolites), the potential toxicity of the metabolites should be included in the available toxicity data. IMI goes through degradation to a large number of metabolites formed by multiple pathways, both

alternative and sequential. For example, after oral administration of ^{14}C-methylene labelled IMI in rats, 95 % of the administered dose was absorbed, with an estimated half-life of 35 min. The maximum concentration of ^{14}C-methylene labelled IMI was reached in blood plasma within 2.5 h after oral or intravenous exposure in rats (Klein and Karl, 1990). Approximately 80% of the administered IMI was excreted in the urine, and 72 % of the urinary secretion was identified as primary metabolite 6CNA and its glycine conjugate (28 % of the total), olefin and 5-hydroxy-IMI (as 30 % of total), and unmodified IMI (15 % of total). In rats exposed orally to ^{14}C-IMI labelled at the 4- and 5- carbon of the imidazolidine ring (Klein and Brauner, 1991), the following metabolites were detected in the urine 48 h after administration: 5-hydroxy-IMI: 13.7 - 14.7 %, olefin-IMI: 7.7-9.1 % and IMI: 6.9 - 14.2 %. Through biotransformation reactions, IMI forms a large number of metabolites (both ionised and non-ionised form) which represent activation and detoxification products relative to action at the nAChRs and possibly more toxic to mammalians than their parent compound (Schulz-Jander *et al.*, 2002; Skandrani *et al.*, 2006). Due to these issues, also IMI TPs were tested during our study for their potential adverse effect on neuronal cell survival. Obtained data relative to TPs cytotoxicity demonstrated significant effects only at highest doses (4000 μM; 1022.4 mg L^{-1}) as parent compound, but this effect was stronger and induced overall cell death, especially in presence of desnitro-IMI (as expected). From a toxicological point of view, the metabolite desnitro-IMI is of particular relevance: (1) this metabolite displays a nicotinic receptor mode of action with a evidently higher toxicity to mammals than IMI, (2) it is a major metabolite produced *in vitro* with human liver microsomes and (3) besides 6CNA, desnitro-IMI is identified as the major degradation product of IMI in the environment (CDPR, 2006).

Furthermore, it was performed also a mutagenic potential screening of IMI, Confidor 200SL and 6CNA with the use of ISO standardised Ames test (ISO 16240, 2005), to provide a full picture of neonicotinoids effects on molecular and cellular mechanisms. Since the introduction of IMI on the insecticide market, its genotoxicity has been studied in different non-target test organisms. Studies showed evidence that exposure to IMI does not increase the micronucleus (MN) frequency in *Vicia faba* seeds, even though it shows a dose – response for DNA damage induction in earthworms *Eisenia fetida* (Zang *et al*., 2000). Increased genotoxicity (MN formation in rats and mutation induction in a bacterial assay) was found in studies which used IMI in combination with an organophosphate (Karabay and Oguz, 2005). Furthermore, one study suggests that IMI and other pesticides may render an organism more susceptible to DNA damage; Shah *et al*. (1997) found that IMI-exposed calf thymus cells had significantly increased amount of damaged DNA than unexposed control cells. On contrary, *in vivo* studies with orally administrated IMI induced no DNA damage. Negative results were obtained in Chinese hamster bone marrow (Herbold, 1989a, b), in mouse germ cells (Volkner, 1990) and no induction of micronuclei formation in mouse (Herbold, 1988). Also, IMI did not cause any DNA damage in bacterial spores (Watanabe, 1990). In our case, neither IMI/Confidor 200SL nor 6CNA presented any indications of possible mutagenic potential.

Different studies relative to IMI performed on mammalian organism models (i.e. rats and mice) were mainly focused on evaluation of LD_{50}, NOEC and LOEC values for conventional endpoints such as: growth, reproduction, survival and behavioural patterns in case of IMI exposure (Table 5 and 6). In general, IMI resulted as moderately toxic to mammals via the oral route of exposure which poses the greatest toxic threat (Mulye, 1996; PMRA, 2001). There are present several studies and a great amount of data on human risk assessment for IMI based on this already established NOEC (NOEL; NOAEL) or LOEC (LOEL; LOAEL) values for conventional endpoints which are summarised in several reports such as: SERA (2006), CDPR (2006) and CCME (2007). Based on these data, U.S. EPA has derived an acute and chronic reference doses (RfD) for IMI of 0.14 and 0.057 mg/kg/day, respectively (US EPA, 2003). Acute and chronic RfDs are well-documented and used directly for all longer term exposures to IMI. Chronic RfD value is based on a NOAEL of 5.7 mg/kg/day in rats (Eiben and Kaliner, 1991; Eiben, 1991) and an uncertainty factor of 100 – two factors of 10 for interspecies and intra-species variability. Acute RfD value is based on a LOAEL of 42 mg/kg in rats (Sheets, 1994) and an uncertainty factor of 300 - a factor of three for extrapolating NOAEL from LOAEL, and two factors of 10 for interspecies and intra-species variability. For workers, the upper range of exposures during the normal broadcast application lead to hazard quotients (HQ) of 1.1, while for general public the highest HQ for non-accidental exposures is 1.5. HQ for accidental exposures associated with spills into a small body of water result in HQs that range from 1.1 (adult male consuming fish) to 15 (child consuming 1 L of contaminated water) (SERA, 2005).

Few data exist on toxicity threshold of pesticides (as pure ingredient, commercial formulation or TP) in humans or specific cell models. Therefore the present study was designed to implement and provide more information regarding IMI and its TPs toxicity on neuronal cell line through examination of several molecular, biochemical and cellular biomarkers (Fig. 49). In addition were provided data on development of NOEC/LOEC values not merely related to conventional endpoints but also to other measured molecular biomarkers. These *in vitro* results support the possibility of potential toxic effects of IMI and its TPs that may induce chronic sensitisation or long-lasting modulation of sensory neurons and molecular/biochemical alteration. This process is of primary importance in several

chronic disorders (typically involving sensory neurons), like skin hypersensitisation, airways hypersensitisation and chronic pain, which might be relevant for occupational exposure. IMI (as nicotine) may induce neuronal plasticity thus changing the sensitivity to chronic sub-threshold levels of this insecticide. Since high potential impact on human health by chronic exposure to pollutants, new molecular findings on alterations at cellular level after IMI/TPs exposure is environmentally and medically relevant, especially if considered for agricultural occupational exposure and toxicology.

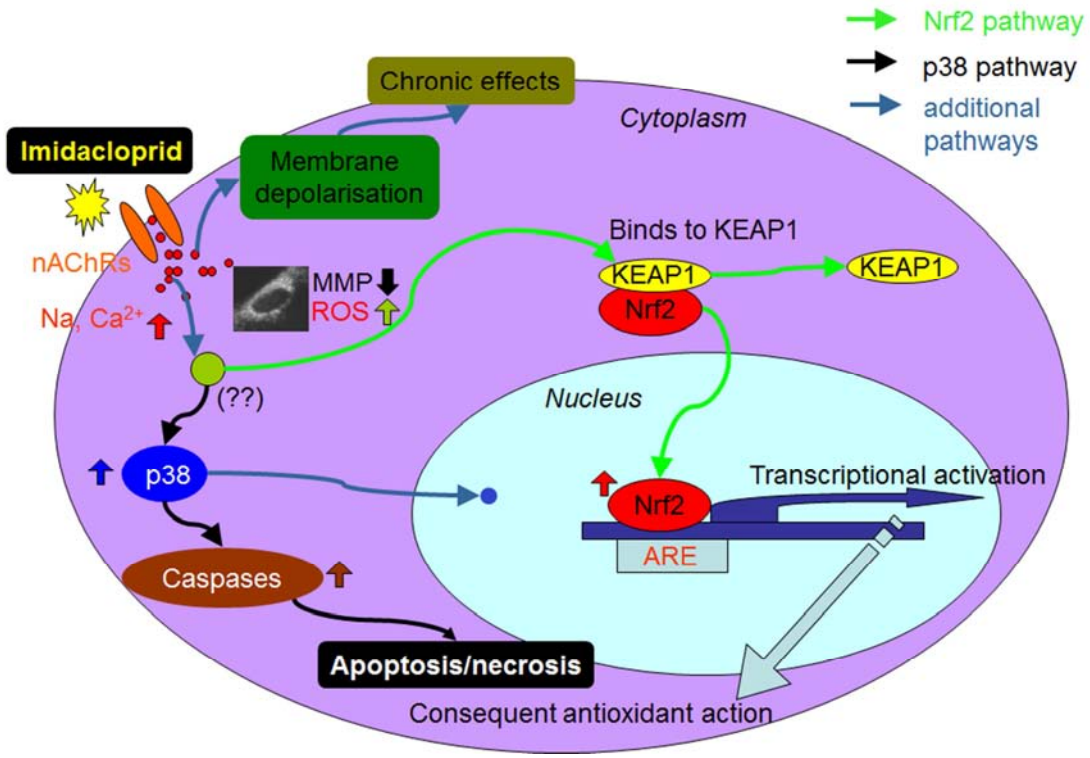


Figure 49: Schematic diagram describing the possible cytotoxic model of IMI and its activated molecular pathways in human neuroblastoma F11 cells. (YNM, 2010 with slight modifications).

In vivo

One problematic of the *in vitro* assays is that they only give information about cellular level, so it is important to integrate the results with different *in vivo* organism models. In our case besides mammalian cell model, were used crustacean *G. fossarum*, freshwater algae *D. subspicatus* and the bacteria *V. fischeri*. Different toxicants act differently and not all living organisms are equally sensible. Consequently, single bioassays will never provide an overall picture of the environmental quality and a representative multiple test battery should be used (Bierksen *et al*., 1998). The use of multi-species laboratory experiments with organisms from different taxonomic and trophic level could provide a possible helpful solution.

Chronic testing was performed on freshwater microalgae *D. subspicatus*. Generally, it appears that algae are some orders of magnitude less sensitive to IMI than arthropod species and exhibiting no effects of IMI on their growth rate (ISO 8692, 2004; Gagliano and Bowers, 1991). Tišler *et al*. (2009) determined for *D. subspicatus* an IC_{50} (72 h) for IMI a. i. at 389 mg L^{-1} (in comparison highest applied concentration in this study was 255 mg L^{-1}). In addition, Kungolos *et al*. (2009) showed that the inhibitory effect of IMI on *Pseudokirchneriella subcapitata* growth at a concentration of 1000 mg L^{-1} was lower than 50 %. Data presented in this research confirmed the same action of IMI as pure compound causing no significant adverse effects on algal growth (Malev *et al.*, 2012). On the contrary, Confidor 200SL was highly toxic to algae due to the presence of co-formulants which started to inhibit their growth already at 0.003 v/v %. On the other hand, 6CNA induced algal growth and proliferation after a 96 h period of exposure at all concentrations, while at the highest dose (157.5 mg L^{-1}) already after 24h induced a significant inhibition and algae death. Presumably the algal growth was inhibited because of the dissociation of the carboxylic group present in 6CNA (Ruiz *et al.*, 2011). This issue induced acidic changes in pH of the algal media and adversely influenced the sensitive microalgae. Algae as primary producers contribute substantially to aquatic habitats and their sensitivity to Confidor 200SL and 6CNA found in this study could cause environmental problems (Malev *et al.*, 2012).

6CNA is a final transformation product formed in environment that does not act as nicotinic agonist but may also contribute to the toxicity effects (Nauen *et al.*, 2001). 6CNA contains the 6-chloropyridinyl moiety and based on its structural/chemical consideration may be of toxicological significance. This transformation product is included in the tolerances established for the IMI residues, although should be considered on its own as other IMI TPs in order to recognise additional IMI-toxicity effects. IMI TPs were already thoroughly presented in the Introduction section, however is important to emphasise again the importance of their formation and presence in environments as they can have possible adverse effects on non-target organisms. Photo-transformation and biotransformation in the aqueous environment appear to be significant transformation routes (Stevens and Halarnkar 1996, as reviewed by Mulye 1997). For example, in a study of Spiteller (1993) was examined radio-labelled IMI degradation in a 30 d laboratory study using water and sediment collected from a pond. By the end of the experiment, 67.6 % of labelled IMI was present in the water (64.0 % as parent IMI and 3.6 % as TPs). In the sediment, 20.4 % was as parent IMI, 0.7 % as TPs, and 8.2 % was as bound residues. On other hand, in soil all processes depend on the soil type and imidazolidin-^{14}C labelled IMI presents a maximum mineralisation to CO_2 of 8.8 % or 14 % after incubation for 12 weeks (Anderson 1995, as reviewed in Mulye 1997). Koskinen *et al*. (2000) found that the bioavailability of IMI decreased in different soils with ageing as indicated by decreases in the amounts of the extracted IMI and increases in the amounts of the bound portion (until 21 d). It is generally noticed that the total ^{14}C recoveries decrease by time, going from 92.9–96.2, 77.1–88.4 to 53.5–62.4 % of the applied IMI at 7, 14 and 21 d after application, respectively (El-Hamady *et al*., 2008). Although ^{14}C-TPs of IMI are rarely detected in soil extracts, the loss of radioactivity must have been due to degradation, strong binding of TPs to the soil matrix and evolution of $^{14}CO_2$. In soil extracts is present unchanged ^{14}C-IMI but also ^{14}C-labelled TPs even if in low percentage/quantity (El-Hamady *et al*., 2008). Until now, only incomplete data on environmental fate and behaviour of such TPs are available, so there is a strong need to further analyse also intermediate degradation products with respect to their environmental behaviour and potential for non-target organism exposure.

In addition to the tested 6CNA a well-known primary IMI degradation product we performed a screening of three more IMI TPs (desnitro-IMI, 5-hydroxy-IMI and olefin-IMI) with ISO standard test based on *V. fischeri* (ISO 11348, 2007). Based on the collected results was possible to establish olefin-IMI as the most harmful to luminescent bacteria (30 min EC_{20} 25.08 mg L^{-1}). However, it was not possible to calculate EC_{50} values for all three TPs due to very low inhibitory effect on bacterial luminescence for given dilution levels. This issue made also impossible to classify the tested compounds within specific toxicity categories based on the EC_{50} values established by the European Commission Directive 93/67/EEC ("*very toxic to aquatic organisms*" [$EC_{50} \leq 1$ mg L^{-1}], "*toxic*" [EC_{50} in the range of 1–10 mg L^{-1}], and "*harmful*" [EC_{50} in the range of 10–100 mg L^{-1}].

Acute toxicity (24 h) of IMI and 6CNA was evaluated on the freshwater amphipod *G. fossarum*. After exposure to the highest dose of IMI (511.3 μg L^{-1}) and 6CNA (315.5 μg L^{-1}), an overall low mortality was noticed. Most significant effect, as in algae, was observed in case of Confidor 200SL. Increased mortality induced by Confidor 200SL supports the idea that major side effects could be caused by additives such as DMSO and NMP. These known co-formulants mixture alone induced no toxicity in amphipods, while the combined action of IMI and co-formulants increased the toxicity of the commercial formulation. In the case of another amphipod crustacean *Hyalella azteca*, Stoughton *et al*. (2008) confirmed its higher sensitivity to formulated product than to technical IMI. Also, other tests have shown formulated pesticides to be more toxic to aquatic organisms (Beggel *et al.*, 2010; Chen *et al.*, 2010; Kaczala *et al.*, 2011). These supplementary substances in commercial formulations often represent the highest proportion in pesticide mixtures, so even a minor concern regarding their toxicity and possible synergistic effects with other ingredients should be considered (Tobiassen *et al.*, 2003; Surgan, 2005). Additional studies will be needed to address the potential effect of additives, but such studies are not easily feasible since identity and quantity of other ingredients is most often regarded as confidential information and therefore rarely revealed in easily accessed literature or product labels.

Neonicotinoids are agonist of nAChRs (Tomizawa and Casida, 2003) and do not exert a direct inhibition of the AChE activity as for example organophosphates. In our study we tested possible indirect inhibitory effects on freshwater amphipods

exposed to neonicotinoids. This measurement was also performed on gills of neonicotinoid exposed mussels and showed an interesting outcome with 'U-shape dynamics' of AChE activity (Dondero *et al.*, 2010). In this study Dondero *et al*. observed significant inhibition at the lowest and at the intermediate tested concentration. On contrary, in our case the outcome of IMI effect on AChE activity presented no indirect effect or changes at all exposure concentrations compared to control group (Malev *et al*., 2012).

It is well-known that pesticides can induce oxidative stress by the generation of ROS, which can induce oxidant-mediated effects (such as increased activities of antioxidant enzymes) and oxidant-mediated toxicities (such as oxidation of lipids) (Zama *et al.*, 2007). Only a few previously published data are available regarding the IMI-induced oxidative stress and these merely relate to mammalian model organisms. These studies showed a slight increase in intracellular ROS and nitric oxide production after IMI exposure (Costa *et al.*, 2009; Duzguner and Erdogan, 2010). A study of Lukančič *et al*. on *G. fossarum* demonstrated that IMI influenced not only the respiration but also the electron transport system (ETS) activity (Lukančič *et al.*, 2010). This effect was a consequence of different processes, including oxidative stress. Partial damage to the inner mitochondrial membrane by lipid peroxidation possibly impaired the function of ETS. For better understanding of ROS involvement in the toxicity mechanisms of neonicotinoids, antioxidant enzyme activity, detoxifying GST mechanism and lipid peroxidative damage were monitored in amphipods. In this study, CAT activity after Confidor 200SL and 6CNA exposure at highest doses was significantly increased and indicated action of the protection mechanisms involved in cellular repair processes. El-Gendy et al. (2010) reported a similar increase of CAT after neonicotinoid exposure, but again only in IMI-treated mice. Enhanced GST activity after Confidor 200SL exposure reflects the detoxification processes in treated gammarids and this induction may be due to the glutathione dependent enzyme system that provides major protection against xenobiotic agents. A recent study on the mosquito, *Aedes aegypti*, demonstrated that exposure to IMI increased glutathione transferase mRNA levels as well as other genes coding for antioxidant proteins (Riaz *et al.*, 2009). In addition was also noticed a slight decrease in antioxidant enzyme GST after exposure to Confidor 200SL (at 255.6 μg L^{-1} of a. i.). This decrease of the GST activity, although not significant was

evident with 1.5-fold lowered GST activity at 255.6 μg L^{-1} of a.i. in Confidor 200SL. This decrease could be interpreted as being overwhelmed by conspicuous ROS production. An additional explanation of enzyme's indirect inhibition is related to their binding with ROS produced also during pesticide metabolism. Metabolism of IMI involves many processes of hydroxylation, i.e. the hydroxylation of the imidazolidine ring at position 4 or 5 leading to the formation of hydroxylated compounds and subsequent loss of important amounts of hydroxyl radicals (Sur and Stork, 2003). Concurrently, with slightly diminished GST activity increased lipid peroxidation levels occurred (at the same exposure concentration of Confidor 200SL). IMI and Confidor 200SL exposure provoked an increase of LP in amphipods. During IMI exposure LP increase occurred at 102.2 μg L^{-1} and was represented by a similar-to-hormetic effect. This increase was induced at lower concentrations of IMI and not at higher doses as expected. On the other hand, Confidor 200SL induced an increase of TBARS products, which was highest at 255.6 μg L^{-1} of a. i. Higher TBARS levels at 255.6 μg L^{-1} suggested that exposure to Confidor 200SL resulted in a different time-course of cellular ROS generation or in a possible direct lipid oxidation due to the interactive action of co-formulants and IMI. It is important to notice potentially different toxicity pathways or time-course effects of the parent compound and its transformation product that were observed during this study. After a 24 h exposure 6CNA provoked strong induction of antioxidant enzyme CAT, while its effect was completely absent on the LP, probably due to highly active CAT. On the contrary, Confidor 200SL altered all parameters confirming its higher toxicity compared to active ingredient.

Behaviour is considered as a useful tool in ecotoxicology since is one of the early warning indicators of toxicant stress (Pestana *et al.*, 2009). During experimental pesticide exposure analysed individual biochemical biomarkers should be linked to behavioural responses whenever this is possible (Hellou, 2011). In this study individuals with modified behaviour were counted. During exposure, animals treated with 511.3 μg L^{-1} (IMI) exhibited an increase in immobility and inactivity that can be a direct IMI effect on neuro-muscular acetylcholine receptors provoking impairment of locomotion and food filtration, with consequent animal starvation and difficulties in ventilation (Férnandez-Casalderrey *et al.*, 1994). Alternatively, 6CNA at the highest dose induced rapid movements and animal hyperactivity, as well as

disorientation. This disoriented behaviour was also shown in non-target organisms, such as *Apis mellifera*. Honey bees treated with IMI were confused and failed to return to their homing site (Bortolotti *et al.*, 2003). Hyperactivity in swimming may also be linked to an avoidance response towards present chemicals (Roast *et al.*, 2000). Interestingly, short-term 6CNA exposure stimulated amphipods moulting processes. Moulting is an essential physiological process for crustaceans controlled by the neuroendocrine system, on which different toxicants, such as pesticides, can act (Waddy *et al.*, 2002). Moreover, moulted or recently moulted animals could be more susceptible to pesticide action.

This *in vivo* research on freshwater amphipod, confirmed the importance of testing commercial formulations of IMI and IMI's transformation products as they interfere with pure compound safety characteristics. Our present results show that commercial formulation of IMI and its by-product 6CNA exert oxidative stress in freshwater amphipods as well as negative effects on algae growth. The induction of CAT, GST and LP levels demonstrates that exposure of *G. fossarum* to Confidor 200SL leads to peroxidation of membrane lipids and triggers antioxidant and detoxifying cellular mechanisms. Amphipods exposed to 6CNA experienced mainly the activation of catalase scavenging protection mechanism. In general, the major toxic effects were due to the commercial formulation Confidor 200SL both in case of algae and amphipods. This issue is relevant as these marketed mixtures are the one applied directly in the environment and should be further monitored.

Different studies and available toxicity data relative to IMI are used for the derivation of specific toxicity values used in environmental risk assessment (SERA, 2006; CCME, 2007. These data separate dose-response assessment in six classes of organisms: terrestrial mammals, birds, non-target terrestrial invertebrates, fish, as well as aquatic invertebrates, and algae which were tested in our study. Acute toxicity values of IMI for aquatic invertebrates have a wide range going from a NOAEC (96 h) of 0.00035 mg L^{-1} for *Hyalella azteca* (England and Bucksath, 1991), to a NOAEC (96 h) of 145 mg L^{-1} for eastern oyster (Wheat and Ward, 1991). On the basis of these studies, NOAEC values of 0.00035 mg L^{-1} and 145 mg L^{-1} are chosen to evaluate acute exposure of sensitive and tolerant aquatic invertebrate species, respectively. On other hand, longer-term studies that assessed reproduction, growth and survival demonstrated *Mysidopsis bahia* as the most sensitive species, with a NOAEC value of 0.000163 mg L^{-1} IMI for growth and reproduction endpoints (Ward, 1991), and *Daphnia magna* as the most tolerant species with a NOAEC (21 d) for immobility of 1.8 mg L^{-1} (Young and Blake, 1990). A 19-week microcosm study conducted as part of EPA's pesticide registration requirements for IMI confirms the sensitivity of amphipods and midges observed in laboratory studies (Moring *et al*., 1992). In addition, on the basis of Moring *et al*. (1992) 19 week microcosm study a recommended regulatory NOAEC for IMI of 0.006 mg L^{-1} was suggested. For freshwater algae risk assessment, NOEC for sensitive species was set at of 6.69 mg L^{-1} based on the study with *Navicula pelliculosa* (Hall, 1996), while for tolerant species was of 119 mg L^{-1} based on the study with *Selenastrum capricornutum* (Gagliano and Bowers, 1991).

As in the human health risk assessment, the environmental risk assessment uses predictive environmental scenarios for an accidental spill that involves the contamination of a small water bodies with IMI. The HQs in these cases for sensitive aquatic invertebrates are extremely high, going from 500 to over 50,000. Although, the probability of such spills may be isolated, these HQ clearly suggest that the greatest risk in the event of an accidental spill will be to aquatic non-target invertebrates (SERA, 2005).

The wish to have estimates of no effect toxicant concentrations is highly desirable and needed, especially in case of non-target organisms. The NOEC, however, can be easily misleading, because of its problematic statistical properties. The interpretation of results from toxicity experiments frequently uses NOECs which are then also applied in human and environmental risk assessments. However this use is under constant criticism due to a substantial statistical problem associated to this concept. Although an alternative is necessary, none of proposed alternative methods to characterise toxic effects is still generally accepted. Therefore, in this study as a selective and supportive tool for evaluation of IMI adverse effects on either *in vitro* or *in vivo* or biological system were evaluated also NOECs and LOECs.

Present *in vivo* study was designed to provide additional information on toxic effects of IMI and its TPs on non-target aquatic organisms and to determine threshold concentrations (i.e. NOECs/LOECs) of these compounds through examination of different endpoints/biomarkers (such as: antioxidant enzymes, LP, survival, growth and behaviour). Interestingly, some recent studies tried to correlate and compare *in vitro* cell toxicity data with the *in vivo* toxicity thresholds reported in literature for aquatic organisms; fish or invertebrates. In particular, human cells kept in culture appeared less sensitive to insecticides than above-mentioned aquatic species. In a study of Skandrani *et al*. (2006) was reported that IMI's LOEC values for fish or invertebrates were of 1.2 and 1.8 μg mL^{-1} while in neuronal and pulmonary human cell lines LOECs were about 100-300 times higher. Similar deductions and significant differences between LOEC values for neuronal cell lines and the ones for invertebrates were confirmed also in our study. Consequently, these organisms seem to be useful for monitoring of aquatic pesticide pollution.

Toxicity testing of pesticides in aquatic environment should be performed on whole organism models at different levels of biological organisation allowing a better comparison of the data. In addition, applying a battery of bioassays and of measured biomarkers we can reflect different species susceptibility to xenobiotics and also the complexity of entire ecosystems. In conclusion, with the increasing use of IMI, this insecticide could be available in the environment during concurrent periods and could pose a possible cumulative risk to non-target organisms (invertebrates, algae, bacteria as well as humans).

CONCLUSIONS

5. Conclusions

- Neuronal cell model applied in this research demonstrated an oxidative stress cellular pattern due to IMI action.

- *V. fischeri* luminescence inhibition confirmed as a suitable biomarker; 6CNA resulted clearly as the most biologically active.

- Algae showed chemically and biologically diverse action of Confidor 200SL (a. i. IMI) and its TP 6CNA; interestingly Confidor 200SL had inhibitory, while 6CNA stimulatory effect on algae growth (hormesis).

- In amphipods as in algae IMI and 6CNA expressed different modes of action; 6CNA affected adversely on whole-cell level not only on neuronal system.

- Behaviour alterations resulted as promising physiological biomarker for neonicotinoid exposure.

- MXR mechanisms (effect on cell membrane level) resulted as a promising and sensitive molecular biomarkers for neonicotinoid exposure; with future standardisation could be a possible fast screening biomarker to range substances based on their biological potential.

- IMI toxicity testing should be performed on whole organism models (*in vivo*) at different levels of biological organisation applying a battery of bioassays and a multi-biomarker approach.

- *In vivo bio*assays should be integrated with *in vitro* (cell model) assays as they give information about IMI effects on cellular level.

- IMI transformation product should be considered on its own in order to recognise additional IMI-toxicity effects and it is reasonable to test these products for hazard identification purposes. A separate assessment of IMI TPs needs to be made, as the physicochemical properties, persistence and toxicity of these compounds may be very different from those of their parent compounds. They may also be present at concentrations similar to or even exceeding the parent compound

- Commercial formulations containing IMI should be also evaluated during comparative toxicity test for potential adverse effect to non-target groups as they are the one applied in the environment and should be considered in detail for human health and environmental risk assessment.

- All toxicity tests relative to IMI should last long enough to compromise the formation of transformation products (degradation products/metabolites) or pesticide “ageing”.

6. ANNEXES

Table 18: Absorbance values and % of cell survival of MTT assay experiments with F11 cells after exposure to IMI, Confidor 200SL and 6CNA for 24 h (A) and 48 h (B). (n=6).

A

MTT assay (F11 cells)

Absorbance values 550 nm

IMIDACLOPRID

24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
1.534	1.5068	1.3068	1.3998	1.3826	1.1818	1.3166	1.2434
1.4745	1.3035	1.2791	1.2776	1.1758	1.2189	1.1707	1.1063
1.4268	1.1481	1.0415	1.1523	1.1423	1.1001	1.2058	1.1793
1.6777	1.7092	1.5328	1.5499	1.6467	1.668	1.5104	1.4771
1.5832	1.452	1.3738	1.2797	1.3913	1.4154	1.485	1.3022
1.4884	1.4124	1.3464	1.3946	1.3614	1.3949	1.4903	1.5373

CONFIDOR 200SL

24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
1.2182	1.2041	1.2117	1.1894	1.1734	1.081	0.98868	0.96044
1.182	1.0828	1.173	1.1372	1.0042	1.0058	1.0896	0.8223
1.2351	1.1546	1.1779	1.1173	1.1461	0.989	1.0639	0.96711
1.2656	1.2783	1.0069	1.3902	1.2455	1.3733	1.1739	0.84955
1.6482	1.3187	1.4856	1.3172	1.2965	1.2479	1.3374	1.282
1.2956	1.2447	1.1904	1.4922	1.3501	1.237	1.3573	1.1784

6CNA

24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
1.2166	1.0528	1.1022	1.1092	1.0563	1.0339	1.1184	1.0903
1.0164	0.99646	0.90174	1.0546	1.0396	1.0072	1.0609	1.1034
0.8212	0.9781	0.93719	0.91524	0.97308	1.0766	1.001	1.1433
1.2475	1.3485	1.4424	1.2992	1.2058	1.2908	1.3183	1.2134
1.4264	1.5517	1.7926	1.7254	1.7999	1.5655	1.4491	1.5748
1.1687	1.3349	1.2354	1.1497	1.2732	1.4061	1.3183	1.1636

% of cell survival

IMIDACLOPRID

24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	98.2269	85.189	91.2516	90.1304	77.0404	85.8279	81.0561
100	88.4028	86.7481	86.6463	79.7423	82.6653	79.3964	75.0288
100	80.4668	72.9955	80.7611	80.0603	77.1026	84.5108	82.6535
100	101.878	91.3632	92.3824	98.1522	99.4218	90.028	88.0432
100	91.713	86.7736	80.83	87.879	89.4012	93.7974	82.2511
100	94.8938	90.4596	93.6979	91.4673	93.7181	100.128	103.154

CONFIDOR 200SL

24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	98.8426	99.4664	97.6359	96.3224	88.7375	81.1591	78.8409
100	91.6074	99.2386	96.2098	84.9577	85.0931	92.1827	69.5685
100	93.4823	95.3688	90.4623	92.7941	80.0745	86.1388	78.3022
100	101.003	79.5591	109.845	98.4118	108.51	92.7544	67.1263
100	80.0085	90.1347	79.9175	78.6616	75.7129	81.1431	77.7818
100	96.0713	91.8802	115.174	104.207	95.477	104.762	90.954

6CNA

24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	86.5362	90.5967	91.1721	86.8239	84.9827	91.9283	89.6186
100	98.0382	88.719	103.758	102.283	99.0948	104.378	108.56
100	119.106	114.124	111.452	118.495	131.101	121.895	139.223
100	108.096	115.623	104.144	96.6573	103.471	105.675	97.2665
100	108.784	125.673	120.962	126.185	109.752	101.591	110.404
100	114.221	105.707	98.3743	108.942	120.313	112.801	99.5636

B

MTT assay (F11 cells)

Absorbance values 550 nm

IMIDACLOPRID

48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
2.5901	2.6651	2.5532	2.5003	2.487	2.4935	2.5575	2.115
2.7437	2.4206	2.4492	2.5065	2.4868	2.1467	2.413	1.7082
2.7055	2.6162	2.7093	2.5411	2.4299	2.3528	2.4768	1.9808
2.3015	2.0315	2.1576	2.2086	2.3867	1.9303	1.6966	1.4175
2.3395	2.1931	2.3032	2.0011	2.1586	2.1216	1.9748	1.3243
2.4635	2.3424	2.4123	2.386	2.2934	2.1754	1.9841	1.5

CONFIDOR 200SL

48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
2.6443	2.6689	2.3459	2.5167	2.3006	2.3173	2.2699	1.2674
2.6555	2.4446	2.244	2.3475	2.2865	1.5745	1.5675	1.0427
2.4745	2.3363	2.1728	2.2437	2.1497	2.1873	1.6504	1.0091
2.5724	2.4159	2.4132	2.3581	2.3107	2.1604	2.0212	1.4177
2.5165	2.1481	2.2779	1.8468	2.0286	1.7089	1.907	1.3817
2.4527	2.1931	2.1741	2.239	2.289	2.2346	1.9663	1.2072

6CNA

48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
2.6133	2.4252	2.2529	2.0113	1.997	1.8436	1.9181	1.7692
2.4027	2.267	2.1852	2.0776	1.9032	1.8666	2.0029	1.9233
2.4012	2.0372	1.9171	1.8864	1.6498	1.5718	1.7937	1.6244
2.4101	2.2093	2.182	2.1409	2.108	2.0925	2.0274	1.8436
2.3035	2.2244	2.0992	2.0868	1.8623	1.9106	1.7915	1.8783
2.1384	1.4488	1.7205	1.3339	1.4097	1.3155	1.4671	1.2807

% of cell survival

IMIDACLOPRID

48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	102.896	98.5753	96.533	96.0195	96.2704	98.7414	81.6571
100	88.2239	89.2663	91.3547	90.6367	78.2411	87.9469	62.259
100	96.6993	100.14	93.9235	89.8133	86.9636	91.5468	73.2138
100	88.2685	93.7476	95.9635	103.702	83.8714	73.7171	61.5903
100	93.7423	98.4484	85.5354	92.2676	90.686	84.4112	56.6061
100	95.0842	97.9217	96.8541	93.0952	88.3053	80.5399	60.889

CONFIDOR 200SL

48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	100.93	88.7153	95.1745	87.0022	87.6338	85.8412	47.9295
100	92.058	84.5039	88.4014	86.1043	59.292	59.0284	39.2657
100	94.415	87.8076	90.6729	86.8741	88.3936	66.6963	40.78
100	93.9162	93.8112	91.6693	89.8266	83.9838	78.5725	55.112
100	85.3606	90.5186	73.3876	80.612	67.9078	75.7799	54.9056
100	89.4157	88.6411	91.2872	93.3257	91.1078	80.1688	49.2192

6CNA

48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	92.8022	86.209	76.964	76.4168	70.5468	73.3976	67.6998
100	94.3522	90.9477	86.4694	79.2109	77.6876	83.3604	80.0474
100	84.8409	79.8392	78.5607	68.7073	65.4589	74.7001	67.6495
100	91.6684	90.5357	88.8303	87.4653	86.8221	84.121	76.4948
100	96.5661	91.1309	90.5926	80.8465	82.9433	77.773	81.5411
100	67.7516	80.4574	62.3784	65.9231	61.518	68.6074	59.8906

Table 19: Absorbance values and % of cell survival of MTT assay experiments with F11 cells after exposure to desnitro-IMI, 5-hydroxy-IMI and olefin-IMI for 24 h (A) and 48 h (B). (n=6).

A

MTT assay (F11 cells)

Absorbance values 550 nm

desnitro-IMI
24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
3.1422	3.3636	3.1918	3.1397	3.1657	3.1371	3.0605	2.8288
3.1643	3.1928	3.1719	3.2005	3.1662	3.175	3.1731	2.2558
3.178	3.2284	3.1804	3.1746	3.1798	3.2273	3.1379	2.4655
3.123	3.334	3.123	3.21	3.165	3.137	3.1657	2.845
3.421	3.321	3.165	3.134	3.145	3.123	3.1662	2.345
3.12	3.432	3.187	3.2413	3.1234	3.1367	3.0798	2.213

5-hydroxy-IMI
24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
3.1572	3.1883	3.2085	3.1854	3.1646	3.1703	2.9085	1.326
3.2061	3.2628	3.1484	3.1594	3.1731	3.1192	3.0806	2.095
3.1416	3.1156	3.1609	3.1565	3.1847	3.1641	3.0579	1.9015
3.1567	3.111	3.201	3.147	3.1567	3.137	3.165	1.425
3.1546	3.123	3.128	3.124	3.111	3.123	2.937	1.982
3.1456	3.147	3.18	3.112	3.201	3.1367	3.0657	2.1784

olefin-IMI
24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
3.166	2.882	3.0786	3.1592	3.2659	3.2019	3.1732	2.3752
3.0261	3.096	3.178	3.1759	3.1777	3.1593	3.0514	2.0955
3.0183	3.0741	3.1852	3.167	3.1791	3.106	2.7317	2.1256
3.176	2.782	3.1786	3.192	3.2659	3.2219	3.1743	2.2752
3.1261	3.076	3.198	3.159	3.1872	3.1494	3.1514	2.0945
3.0983	3.1441	3.1652	3.167	3.1797	3.116	2.75147	2.1275

desnitro-IMI
24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	103.166	100.628	100.32	100.287	100.291	98.8086	79.6046
100	103.127	97.8599	96.2626	97.0597	96.1829	93.8343	86.7304
100	97.8906	97.2498	98.1267	97.0751	97.3449	97.2866	69.1624
100	98.9821	97.5104	97.3326	97.492	98.9484	96.2074	75.5917
100	102.22	95.7506	98.418	97.0383	96.1798	97.0597	87.2271
100	101.821	97.0383	96.0878	96.4251	95.7506	97.0751	71.8972

5-hydroxy-IMI
24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	100.65	100.136	99.9621	100.184	99.4603	95.1825	55.9974
100	102.983	99.3719	99.7191	100.152	98.4503	97.232	66.1238
100	98.3366	99.7664	99.6276	100.518	99.8674	96.5155	60.0164
100	98.1915	101.032	99.3277	99.6339	99.0121	99.8958	44.9768
100	98.5702	98.728	98.6018	98.1915	98.5702	92.6996	62.5572
100	99.3277	100.369	98.223	101.032	99.0026	96.7617	68.7561

olefin-IMI
24 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	95.4491	99.5583	100.194	101.466	99.8261	94.439	69.5542
100	100.843	103.513	103.445	103.504	102.904	99.3898	68.2544
100	100.129	103.748	103.155	103.549	101.168	88.9766	69.2348
100	90.615	103.533	103.969	106.376	104.943	103.393	74.1075
100	100.191	104.165	102.895	103.813	102.582	102.647	68.2218
100	102.409	103.096	103.155	103.569	101.494	89.6205	69.2967

B

MTT assay (F11 cells)

Absorbance values 550 nm

desnitro-IMI
48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
3.2228	3.3682	3.1855	3.2054	3.2321	2.133	1.0293	0.66286
3.1998	3.1926	3.155	3.1863	3.1699	3.1673	2.894	0.56907
3.2029	3.1781	3.1889	3.1786	3.174	3.1722	3.2313	0.59862
3.2328	3.546	3.1335	3.2051	3.2341	2.103	1.0193	0.6686
3.1548	3.1846	3.134	3.1663	3.1674	3.153	2.794	0.5407
3.2089	3.1651	3.1619	3.1762	3.1274	3.1922	3.1263	0.58262

5-hydroxy-IMI
48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
3.1807	3.0974	3.2092	3.1785	3.1871	2.8908	2.9582	0.77138
3.3291	3.2984	3.19	3.1679	3.1814	3.1819	2.2017	0.71477
3.1511	3.1738	3.1522	3.1501	3.1471	3.0852	2.8612	1.0027
3.1827	3.094	3.292	3.1685	3.181	2.8708	2.9762	0.778
3.3143	3.283	3.123	3.1699	3.1904	3.1769	2.2019	0.71457
3.1501	3.1728	3.1502	3.1461	3.151	3.0452	2.7822	1.2027

olefin-IMI
48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
3.187	3.2021	3.1601	3.1535	3.2824	3.1692	3.1554	0.66549
3.3054	3.1553	3.1953	3.1409	3.1365	3.1832	3.1525	0.71075
3.1267	3.1098	3.155	3.1691	3.1557	3.1498	3.1275	0.9881
3.1101	3.161	3.182	3.1409	3.108	3.0925	3.0274	0.8436
3.3035	3.1853	2.992	3.1868	3.2623	3.106	3.1915	0.8782
3.1384	3.155	3.1205	3.1339	3.1097	3.155	3.1671	0.6807

% of cell survival

desnitro-IMI
48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	101.178	99.0016	99.4265	99.4857	88.0214	74.3296	19.0177
100	99.5044	98.3326	99.3081	98.7969	98.7159	90.1979	17.7363
100	99.0525	99.3891	99.0681	98.9247	98.8686	100.711	18.6573
100	110.519	97.6625	99.894	100.798	65.5446	31.7687	20.8384
100	99.2551	97.678	98.6847	98.719	98.2702	87.0812	16.8521
100	98.6473	98.5476	98.9933	97.4723	99.492	97.4381	18.1586

5-hydroxy-IMI
48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	99.055	98.8666	98.2983	98.496	94.7935	83.0264	29.9025
100	102.425	99.0591	98.3728	98.792	98.8076	68.3694	32.1925
100	98.556	97.8853	97.8201	97.7269	95.8047	88.8489	31.1369
100	96.078	102.227	98.3915	98.7796	89.147	92.42	35.0403
100	101.947	96.9785	98.4349	99.0715	98.6523	68.3756	32.1835
100	98.525	97.8232	97.6959	97.848	94.5626	86.3957	37.3475

olefin-IMI
48 h

control	1 µM	30 µM	100 µM	200 µM	500 µM	1000 µM	4000 µM
100	98.4209	98.87	98.3824	99.5374	98.7847	98.0903	24.5796
100	98.4073	99.6549	97.9582	97.821	99.2775	98.32	22.1668
100	96.9883	98.398	98.8377	98.4198	98.2358	97.5403	30.8168
100	98.5851	99.2401	97.9582	96.9321	96.4487	94.4184	26.3102
100	99.343	93.3143	99.3898	101.744	96.8698	99.5363	27.3893
100	98.398	97.322	97.7399	96.9852	98.398	98.7754	21.2296

Table 20: Absorbance values and % of cell survival of MTT assay experiments with F11 cells after pre-treatment (30 min) with p38 inhibitor SB203580, vitamin C (NAC) and mixture of tocopherols (vitamin E) in presence of 1000 µM (255.6 mg L^{-1}) and 4000 µM (1022.4 mg L^{-1}) IMI for 24 h (A) and 48 h (B). (n=6).

A

MTT assay (F11 cells)

Absorbance values 550 nm

p38 inhibitor (SB203580)

24 h

control	DMSO	DMSO+NMP	SB203580+ 1000 µM IMI	SB203580+ 4000 µM IMI
0.372	0.41	0.427	0.439	0.431
0.539	0.476	0.473	0.359	0.539
0.387	0.395	0.421	0.379	0.442
0.455	0.492	0.41	0.315	0.543
0.455	0.41	0.41	0.315	0.54
0.487	0.45	0.42	0.439	0.53

vitamin E

24 h

control	DMSO	DMSO+NMP	vitamin E+1000 µM IMI	vitamine E+4000 µM IMI
0.372	0.41	0.427	0.415	0.431
0.539	0.476	0.473	0.433	0.539
0.387	0.395	0.421	0.259	0.442
0.455	0.492	0.41	0.395	0.543
0.455	0.41	0.41	0.415	0.543
0.487	0.45	0.42	0.395	0.44

NAC

24 h

control	DMSO	DMSO+NMP	NAC+1000 µM IMI	NAC+4000 µM IMI
0.372	0.41	0.427	0.369	0.431
0.539	0.476	0.473	0.483	0.539
0.387	0.395	0.421	0.329	0.442
0.455	0.492	0.41	0.342	0.543
0.455	0.41	0.41	0.342	0.45
0.487	0.45	0.42	0.369	0.53

% of cell survival

p38 inhibitor (SB203580)

24 h

control	DMSO	DMSO+NMP	SB203580+ 1000 µM IMI	SB203580+ 4000 µM IMI
100	93.56	97.48	100.22	101.63
100	108.67	107.99	81.9	149.38
100	90.18	96.11	86.25	92.21
100	112.32	93.06	71.91	108.4
100	93.56	93.06	71.91	151.2
100	94	94.01	100.22	149.5

vitamine E

24 h

control	DMSO	DMSO+NMP	vitamin E+1000 µM IMI	vitamine E+4000 µM IMI
100	93.56	97.48	94.74	117.41
100	108.67	107.99	98.85	174.79
100	90.18	96.11	59.13	129.5
100	112.32	93.06	90.18	90.16
100	93.56	93.06	94.74	90.16
100	94	94.01	59.13	117.6

NAC

24 h

control	DMSO	DMSO+NMP	NAC+1000 µM IMI	NAC+4000 µM IMI
100	93.56	97.48	84.24	96.72
100	108.67	107.99	110.2	81.9
100	90.18	96.11	75.11	112.2
100	112.32	93.06	78.02	84.42
100	93.56	93.06	78.02	82
100	94	94.01	84.24	112.8

B

MTT assay (F11 cells)

Absorbance values 550 nm

p38 inhibitor (SB203580)

48 h

control	DMSO	DMSO+NMP	SB203580+ 1000 µM IMI	SB203580+ 4000 µM IMI
1.162	1.296	1.423	1.059	0.801
1.287	1.193	1.202	1.024	0.825
1.24	1.202	1.183	0.947	0.73
1.24	1.198	1.235	1.043	0.828
1.164	1.201	1.236	1.045	0.835
1.288	1.294	1.424	1.05	0.802

vitamine E

48 h

control	DMSO	DMSO+NMP	vitamin E+1000 µM IMI	vitamine E+4000 µM IMI
1.162	1.296	1.423	0.936	0.864
1.287	1.193	1.202	1.202	0.876
1.24	1.202	1.183	1.36	0.677
1.24	1.198	1.235	1.049	0.819
1.164	1.201	1.236	1.2	0.822
1.288	1.294	1.424	0.93	0.823

NAC

48 h

control	DMSO	DMSO+NMP	NAC+1000 µM IMI	NAC+4000 µM IMI
1.162	1.296	1.423	1.37	0.803
1.287	1.193	1.202	1.234	0.75
1.24	1.202	1.183	0.915	0.868
1.24	1.198	1.235	1.2	0.875
1.164	1.201	1.236	1.2	0.88
1.288	1.294	1.424	1.3	0.87

% of cell survival

p38 inhibitor (SB203580)

48 h

control	DMSO	DMSO+NMP	SB203580+ 1000 µM IMI	SB203580+ 4000 µM IMI
100	105.19	115.5	85.95	67.07
100	96.83	97.56	83.11	69.08
100	97.56	96.02	76.86	61.12
100	97.24	100.24	84.65	69.33
100	97.65	100.3	84.7	70.02
100	105.1	100.7	84.92	67.08

vitamine E

48 h

control	DMSO	DMSO+NMP	vitamin E+1000 µM IMI	vitamine E+4000 µM IMI
100	105.19	115.5	75.97	72.34
100	96.83	97.56	97.56	73.35
100	97.56	96.02	110.38	56.68
100	97.24	100.24	85.146	68.57
100	97.65	100.3	97.7	68.7
100	105.1	100.7	76.02	68.7

NAC

48 h

control	DMSO	DMSO+NMP	NAC+1000 µM IMI	NAC+4000 µM IMI
100	105.19	115.5	111.2	67.23
100	96.83	97.56	100.16	62.8
100	97.56	96.02	74.26	72.68
100	97.24	100.24	97.4	73.26
100	97.65	100.3	97.4	74.12
100	105.1	100.7	98.1	73.45

Table 21: Mean values relative to p38 signal intensity quantification (grey values of immunopositive signals after background – BG subtraction) after exposure to 400 µM (102.2 mg L^{-1}) and 4000 µM (1022.4 mg L^{-1}) IMI for 30 min. (n=3).

p38

CONTROL

	No. of cell	whole	BG1	BG2	BG3	mean BG		
1	16	25.712	21.679	25.237	21.927	22.94767	2.76433	0.172771
2	23	26.841	24.943	19.467	26.104	23.50467	3.33633	0.145058
3	27	28.423	24.413	31.475	19.748	25.21200	3.21100	0.118926
4	27	24.797	28.104	19.543	20.658	22.76833	2.02867	0.075136
5	20	25.758	18.341	29.615	16.768	21.57467	4.18333	0.209167
6	30	26.34	23.735	27.089	16.871	22.56500	3.77500	0.125833
7	32	25.21	26.114	25.141	18.485	23.24667	1.96333	0.061354
8	22	22.358	25.208	24.409	13.508	21.04167	1.31633	0.059833
9	46	30.138	26.102	28.557	17.929	24.19600	5.94200	0.129174
10	24	30.523	23.897	32.656	26.857	27.80333	2.71967	0.113319

400 µM IMI

	No. of cell	whole	BG1	BG2	BG3	mean BG		
1	16	27.04	18.366	31.274	22.559	24.06633	2.97367	0.185854
2	34	34.9	31.417	37.82	24.962	31.39967	3.50033	0.102951
3	20	28.307	29.774	21.316	21.529	24.20633	4.10067	0.205033
4	30	26.041	27.992	19.943	26.747	24.89400	1.14700	0.038233
5	29	29.458	20.048	33.697	24.991	26.24533	3.21267	0.110782
6	24	25.858	13.657	26.757	17.289	19.23433	6.62367	0.275986
7	18	25.142	17.169	27.379	16.638	20.39533	4.74667	0.263704
8	16	23.301	25.353	14.14	24.927	21.47333	1.82767	0.114229
9	17	26.934	20.588	27.739	19.138	22.48833	4.44567	0.26151
10	19	26.829	18.284	19.587	30.582	22.81767	4.01133	0.211123

4000 µM IMI

	No. of cell	whole	BG1	BG2	BG3	mean BG		
1	42	28.964	22.312	27.366	20.829	23.50233	5.46167	0.13004
2	38	35.155	29.39	32.309	22.476	28.05833	7.09667	0.186754
3	29	34.938	32.034	25.658	24.459	27.38367	7.55433	0.260494
4	18	38.737	30.588	29.085	23.939	27.87067	10.86633	0.603685
5	28	30.375	29.084	28.796	20.626	26.16867	4.20633	0.150226
6	22	32.466	25.677	23.85	29.177	26.23467	6.23133	0.283242
7	25	61.177	59.357	50.707	50.939	53.66767	7.50933	0.300373
8	30	32.237	27.696	24.61	29.89	27.39867	4.83833	0.161278
9	21	29.744	19.644	24.006	29.196	24.28200	5.46200	0.260095
10	30	31.173	18.466	29.62	18.311	22.13233	9.04067	0.301356

Table 22: Mean values relative to ERK signal intensity quantification (grey values of immunopositive signals after background – BG subtraction) after exposure to 400 µM (102.2 mg L^{-1}) and 4000 µM (1022.4 mg L^{-1}) IMI for 30 min. (n=3).

ERK

CONTROL

	No. of cell	whole	BG1	BG2	BG3	mean BG		
1	82	17.859	7.47	7.851	6.712	7.34433	10.51467	0.089565
3	60	8.141	7.795	7.91	5.491	7.06533	1.07567	0.117756
5	40	7.809	7.541	6.988	5.336	6.62167	1.18733	0.165542
6	36	10.303	7.513	6.01	7.13	6.88433	3.41867	0.191231
8	50	14.061	7.661	6.214	8.032	7.30233	6.75867	0.146047
9	46	9.938	5.832	6.823	5.955	6.20333	3.73467	0.134855

400 µM IMI

	No. of cell	whole	BG1	BG2	BG3	mean BG		
1	25	17.301	7.338	11.473	10.733	9.84800	7.45300	0.39392
2	25	15.521	10.637	9.896	6.623	9.05200	6.46900	0.36208
3	28	16.977	8.395	10.743	7.638	8.92533	8.05167	0.318762
4	31	15.139	11.125	11.962	7.527	10.20467	4.93433	0.329183
5	22	14.614	7.062	11.473	7.528	8.68767	5.92633	0.394894
6	36	17.299	7.927	11.208	7.754	8.96300	8.33600	0.248972
7	25	13.434	9.962	10.009	7.064	9.01167	4.42233	0.360467
9	29	20.264	9.411	8.373	11.119	9.63433	10.62967	0.332218
10	30	22.399	12.439	7.767	10.807	10.33767	12.06133	0.344589

4000 µM IMI

	No. of cell	whole	BG1	BG2	BG3	mean BG		
1	27	12.439	10.613	10.322	7.08	9.40500	3.63400	0.348333
2	51	18.657	11.775	9.057	11.478	10.43067	8.243333	0.204523
3	32	12.094	10.851	9.828	6.577	9.08533	3.00867	0.283917
4	34	14.964	10.127	10.333	6.579	9.01300	5.95100	0.265088
5	36	17.834	9.403	10.838	6.581	8.94067	8.89333	0.248352
5	27	12.439	10.813	10.322	7.08	9.40500	3.03400	0.348333
6	51	18.687	11.755	8.059	11.478	10.43067	8.25633	0.204523
7	52	18.687	11.855	8.059	11.578	10.43067	8.25633	0.20059
8	27	13.6	10.101	9.423	10.246	9.92333	3.67667	0.367531
9	41	18.574	14.768	13.448	15.932	14.71600	3.85800	0.358927
10	46	13.594	11.74	10.286	11.54	11.18867	2.40533	0.243232

Table 23: Mean values relative to Nrf2 signal intensity quantification (grey values of immunopositive signals after background – BG subtraction) after exposure to 4000 µM (1022.4 mg L^{-1}) IMI for 30 and 240 min (n=3).

Nrf2

control; 24h

	cell No.	cell brightness Nrf2(mean)	cell BG1	cell BG2	cell BG3
1	42	44.937	34.889	35.784	35.254
2	55	49.608	32.332	31.295	30.006
3	54	77.607	49.516	50.382	48.519
4	70	60.476	43.369	36.046	44.796
5	68	56.913	42.287	33.698	37.458
6	32	86.482	70.93	70.099	72.001
7	22	88.226	66.632	72.145	68.774
8	46	55.404	44.973	36.903	37.184
9	54	53.72	41.746	32.399	46.98
10	42	65.884	56.385	53.043	55.499

Mean BG	Nrf2	Nrf2/cell
35.309	9.628	0.229238095
31.211	18.397	0.334490909
49.47233333	28.13466667	0.521012346
41.40366667	19.07233333	0.272461905
37.81433333	19.09866667	0.280862745
71.01	15.472	0.4835
69.18366667	19.04233333	0.865560606
39.68666667	15.71733333	0.341681159
40.375	13.345	0.24712963
54.97566667	10.90833333	0.259722222

4000 µM; 30 min

	cell No.	cell brightness Nrf2(mean)	cell BG1	cell BG2	cell BG3
1	36	56.109	48.124	36.679	46.437
2	25	58.418	41.141	46.341	42.499
3	58	67.952	39.791	39.398	53.114
4	32	109.803	97.237	104.235	99.341
5	24	53.038	46.358	37.933	37.836
6	46	62.136	41.516	49.104	41.237
7	35	58.025	40.584	49.52	39.922
8	31	52.866	36.302	42.351	38.895
9	26	54.562	35.297	40.729	40.679
10	25	63.755	45.383	51.91	48.82

Mean BG	Nrf2	Nrf2/cell
43.74666667	12.36233333	0.343398148
43.327	15.091	0.60364
44.101	23.851	0.411224138
100.271	9.532	0.297875
40.709	12.329	0.513708333
43.95233333	18.18366667	0.395297101
43.342	14.683	0.419514286
39.18266667	13.68333333	0.441397849
38.90166667	15.66033333	0.602320513
48.70433333	15.05066667	0.602026667

4000 µM; 240 min

	cell No.	cell brightness Nrf2(mean)	cell BG1	cell BG2	cell BG3
1	14	86.641	75.978	85.3	85.209
2	43	70.849	52.303	49.266	57.792
3	21	64.554	54.483	47.043	50.365
4	17	61.688	51.361	53.95	53.338
5	25	68.01	55.05	59.536	53.832
7	41	69.22	51.557	51.641	49.535
8	30	141.842	132.231	138.322	118.34
9	27	64.673	61.644	50.217	50.077
10	26	68.804	65.998	55.201	62.475

Mean BG	Nrf2	Nrf2/cell
82.16233333	4.478666667	0.319904762
53.12033333	17.72866667	0.412294574
50.63033333	13.92366667	0.663031746
52.883	8.805	0.517941176
56.13933333	11.87066667	0.474826667
50.911	18.309	0.446560976
129.631	12.211	0.407033333
53.97933333	10.69366667	0.396061728
61.22466667	7.579333333	0.291512821

Table 24: Mean values relative to JC-1 signal intensity quantification (JC-1 green-red signal ratio) and % of cells with higher MMP (red signal) after exposure to 1000 µM (255.6 mg L^{-1}) and 4000 µM (1022.4 mg L^{-1}) IMI for 15 and 60 min (n=3).

JC-1

Control

sample n.	ALL CELLS	red signal	% cells (higher MMP)
1	110	24	21.8
2	106	36	33.96
3	89	26	29.21
4	139	64	46.04
5	115	60	52.17
6	105	54	51.42
7	109	38	34.86
8	118	88	74.57
9	131	48	36.64
10	47	27	57.44
1	54	29	53.7
2	53	17	32.07
3	97	28	28.86
4	125	63	50.04
5	118	72	61

Control

sample n.	ALL CELLS	red signal	% cells (higher MMP)
1	61	6	9.83
2	72	13	18.05
3	81	13	16.04
4	60	11	18.33
5	100	12	12
6	97	18	18.55
7	76	16	21.05
8	67	25	37.31
9	69	20	28.98
10	70	10	14.28
1	64	9	14.06
2	66	40	60.6
3	86	43	50
4	82	38	46.34
5	114	54	47.36

4000 µM; 15 min

sample n.	ALL CELLS	red signal	% cells (higher MMP)
1	12	7	58.3
2	49	12	24.48
3	75	4	5.33
4	35	15	42.85
5	55	25	45.45
6	36	16	44.44
7	43	13	30.23
8	42	13	30.95
9	43	13	30.23
10	79	14	17.27
1	108	10	9.25
2	47	14	29.78
3	31	12	38.7
4	36	12	33.33
5	19	8	42.1

4000 µM; 15 min

sample n.	ALL CELLS	red signal	% cells (higher MMP)
1	94	19	20.21
2	95	30	31.57
3	109	13	11.92
4	37	1	2.7
5	103	17	16.5
6	108	9	8.33
7	92	18	19.56
8	76	18	23.68
9	46	20	43.47
10	81	14	17.28
1	73	7	9.58
2	51	10	19.6
3	98	16	16.32
4	44	20	45.45
5	87	25	28.73

4000 µM; 60 min

sample n.	ALL CELLS	red signal	% cells (higher MMP)
1	52	12	22.64
2	42	18	42.85
3	45	17	37.77
4	24	9	37.5
5	17	6	35.29
6	14	11	78.57
7	35	13	37.14
8	20	6	30
9	31	9	29.03
10	43	13	30.23
1	60	39	65
2	57	23	40.35
3	90	30	33.33
4	69	34	49.27
5	74	24	32.43

4000 µM; 60 min

sample n.	ALL CELLS	red signal	% cells (higher MMP)
1	99	9	9.09
2	29	10	34.48
3	63	11	17.46
4	38	13	34.21
5	69	12	17.39
6	83	5	6.02
7	108	11	10.18
8	83	6	7.22
9	56	10	17.85
10	90	7	7.77
1	90	6	6.66
2	60	5	8.33
3	61	6	9.83
4	82	10	12.19
5	69	7	10.14

Table 25: CAT activity in F11 cells exposed to 1000 µM (255.6 mg L^{-1}) and 4000 µM (1022.4 mg L^{-1}) IMI for 24 and 48 h. Representative absorbance (AU) values of whole 2 min measurement (selected for every 30 s) and data normalised on total protein content (µmol/min/mg protein). (n=3).

CAT

24 h

Control

time	*AU*	*AU*	*AU*
0 s	0.008508	0.4167	0.1138
30 s	0.007056	0.41096	0.11353
60 s	0.00676	0.40603	0.11289
90 s	0.007455	0.40235	0.11262
120 s	0.006561	0.40038	0.11225

1000 µM IMI

time	*AU*	*AU*	*AU*
0 s	0.014257	0.30639	0.06278
30 s	0.014281	0.30271	0.06139
60 s	0.013046	0.29939	0.06156
90 s	0.013548	0.29705	0.06026
120 s	0.012263	0.29519	0.05979

4000 µM IMI

time	*AU*	*AU*	*AU*
0 s	0.166809	0.24196	0.05851
30 s	0.163384	0.23991	0.05749
60 s	0.16239	0.23788	0.05719
90 s	0.160803	0.23615	0.05735
120 s	0.160877	0.23494	0.05687

Calculated values normalised on protein content

24 h	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**
control	0.051	0.0257	1.98
1000 µM	0.038	0.0256	1.48
4000 µM	0.15	0.0184	8.15
control	0.028	0.0862	0.32
1000 µM	0.294	0.0604	4.86
4000 µM	0.184	0.048	3.83
control	0.04	0.0429	0.93
1000 µM	0.025	0.049	0.51
4000 µM	0.043	0.0448	0.88

48 h

Control

time	*AU*	*AU*	*AU*
0 s	0.033807	0.16851	0.19837
30 s	0.03209	0.1638	0.19618
60 s	0.032174	0.16417	0.19524
90 s	0.032248	0.16719	0.1947
120 s	0.032269	0.1637	0.19429

1000 µM IMI

time	*AU*	*AU*	*AU*
0 s	0.017433	0.09331	0.03723
30 s	0.017026	0.09245	0.03701
60 s	0.016976	0.09228	0.03673
90 s	0.016976	0.09249	0.03656
120 s	0.016057	0.09218	0.03612

4000 µM IMI

time	*AU*	*AU*	*AU*
0 s	0.018725	0.09583	0.1073
30 s	0.018338	0.09563	0.10771
60 s	0.018511	0.09403	0.10556
90 s	0.018593	0.09385	0.10419
120 s	0.018522	0.09373	0.10348

Calculated values normalised on protein content

48 h	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**
control	0.04	0.032	1.25
1000 µM	0.014	0.0378	0.37
4000 µM	0.005	0.0242	0.2
control	0.049	0.041	1.19
1000 µM	0.073	0.0388	1.88
4000 µM	0.133	0.02	6.65
control	0.034	0.111	0.3
1000 µM	0.139	0.024	5.79
4000 µM	0.076	0.022	3.45

Table 26: Absorbance (AU) values for LP measurements in F11 cells exposed to 1000 µM (255.6 mg L^{-1}) and 4000 µM (1022.4 mg L^{-1}) IMI for 24 and 48 h and data normalised on total protein content (nmol//mg protein of MDA). (n=3).

LP

24 h

	AU 535 nm	AU 600 nm	AU 535-600 nm
control	0.02	0.012	0.008
control	0.005	0.001	0.004
control	0.009	0.002	0.007
control	0.006	0.001	0.005
control	0.003	0.001	0.002
control	0.007	0.001	0.006
1000 µM	0.031	0.01	0.021
1000 µM	0.032	0.011	0.021
1000 µM	0.051	0.003	0.048
1000 µM	0.022	0.004	0.018
1000 µM	0.045	0	0.045
1000 µM	0.014	0.001	0.013
4000 µM	0.46	0.005	0.455
4000 µM	0.41	0.003	0.407
4000 µM	0.077	0.002	0.075
4000 µM	0.059	0.005	0.054
4000 µM	0.209	0.003	0.206
4000 µM	0.313	0.004	0.309

MDA nmol/ml medium

control	1000 µM	4000 µM
1.05	2.4	18.45
0.375	2.7	56.25
0.75	0.75	2.25
0.375	0.45	9
1.05	2.55	1.8
0.27	1.8	2.55

normalised MDA ng/mg protein

15.69	13.333	87.857
8.09	24.545	*274*
3.703	7.5	16.071
1.923	7.32	45
19.02	14.488	7.826
1.313	14.285	19.318

protein (mg)

0.2355	0.2595	*0.067*
0.33	0.2595	0.124
0.3525	0.1575	0.2445
0.1815	0.1785	0.23

48 h

	AU 535 nm	AU 600 nm	AU 535-600 nm
control	0.006	0.01	0.005
control	0.01	0.007	0.003
control	0.005	0.001	0.004
control	0.003	0	0.003
control	0.008	0.003	0.005
control	0.002	0	0.002
1000 µM	0.019	0.007	0.012
1000 µM	0.02	0.006	0.014
1000 µM	0.005	0.001	0.004
1000 µM	0.005	0.001	0.004
1000 µM	0.013	0.004	0.011
1000 µM	0.011	0.002	0.009
4000 µM	0.102	0.01	0.092
4000 µM	0.423	0.01	0.413
4000 µM	0.014	0.002	0.012
4000 µM	0.058	0.006	0.052
4000 µM	0.013	0.004	0.009
4000 µM	0.015	0.002	0.013

MDA nmol/ml medium

control	1000 µM	4000 µM
1.05	2.4	18.45
0.375	2.7	56.25
0.75	0.75	2.25
0.375	0.45	9
1.05	2.55	1.8
0.27	1.8	2.55

normalised MDA ng/mg protein

15.69	13.333	87.857
8.09	24.545	*274*
3.703	7.5	16.071
1.923	7.32	45
19.02	14.488	7.826
1.313	14.285	19.318

0.0669	0.018	0.21
0.04635	0.011	*0.205*
0.2025	0.01	0.14
0.195	*0.0614*	0.2

Table 27: Values of luminescence measurements (L_o and L_e) and % of inhibition in **V. fischeri** *exposed for 30 min to aqueous solutions of desnitro-IMI, olefin-IMI and 5-hydroxy-IMI.*

V. fischeri

desnitro-IMI

Geometric dillution
Concentration: 100 mg L^{-1}

mg L^{-1}	*G-value*	*Lo*	*Le*			*% Inhibition average*	*Valid*
0	K	349,10	323,10	318,2368	-1,5		1,53
		397,70	357,00	362,5402	1,5		Yes
0.39	512	370,30	325,10	337,5626	3,7		0,58
		363,30	315,10	331,1814	4,9	4,27	Yes
0.78	256	336,40	272,30	306,6596	11,2		0,58
		364,30	304,60	332,093	8,3	9,74	Yes
1.5625	128	344,00	299,30	313,5877	4,6		0,25
		345,00	298,60	314,4993	5,1	4,81	Yes
3.125	64	361,20	311,00	329,2671	5,5		0,58
		339,00	295,50	309,0297	4,4	4,96	Yes
6.25	32	355,10	308,30	323,7064	4,8		1,00
		316,10	268,70	288,1543	6,8	5,76	Yes
12.5	16	338,40	279,70	308,4828	9,3		0,05
		329,10	271,70	300,005	9,4	9,38	Yes
25	8	323,00	249,10	294,4443	15,4		0,41
		312,30	243,20	284,6902	14,6	14,99	Yes
50	4	302,10	220,90	275,392	19,8		0
		312,80	228,70	285,146	19,8	19,79	Yes
100	2	332,30	190,00	302,9221	37,3		0,68
		339,30	198,20	309,3032	35,9	36,60	Yes

olefin-IMI

Geometric dillution
Concentration: 100 mg L^{-1}

mg L^{-1}	*G-value*	*Lo*	*Le*			*% Inhibition average*	*Valid*
0	K	667,50	695,70	667,7769	-4,2		4,18
		719,50	689,70	719,7984	4,2		no
0.39	512	703,00	668,70	703,2916	4,9		2,66
		688,30	691,40	688,5855	-0,4	2,25	Yes
0.78	256	672,40	660,80	672,6789	1,8		0,36
		656,10	640,00	656,3721	2,5	2,13	Yes
1.5625	128	691,30	646,20	691,5867	6,6		0,18
		698,60	650,50	698,8898	6,9	6,74	Yes
3.125	64	735,80	699,20	736,1052	5,0		2,05
		716,10	651,10	716,397	9,1	7,06	Yes
6.25	32	728,40	667,00	728,7021	8,5		2,53
		730,40	631,90	730,7029	13,5	10,99	Yes
12.5	16	726,50	655,80	726,8013	9,8		2,73
		707,60	600,10	707,8935	15,2	12,50	Yes
25	8	761,30	601,20	761,6158	21,1		0,67
		689,80	554,00	690,0861	19,7	20,39	Yes
50	4	712,30	503,70	712,5954	29,3		1,58
		708,10	523,10	708,3937	26,2	27,74	Yes
100	2	690,00	388,10	690,2862	43,8		0,56
		701,40	386,70	701,6909	44,9	44,33	Yes

Table 27: Values of luminescence measurements (L_o and L_e) and % of inhibition in* V. fischeri *exposed for 30 min to aqueous solutions of desnitro-IMI, olefin-IMI and 5-hydroxy-IMI (continued).

V. fischeri

5-hydroxy-IMI

Geometric dillution
Concentration: 100 mg/L

mg L $^{-1}$	*G-value*	*Lo*	*Le*	*Mean*		*% Inhibition average*	*Valid*
0	K	801,50	841,60	836,9317	-0,6		0,56
		825,90	857,60	862,4104	0,6		Yes
0.39	512	815,70	809,00	851,7595	5,0		0,98
		801,40	778,40	836,8273	7,0	6,00	Yes
0.78	256	831,70	803,40	868,4668	7,5		0,37
		841,90	819,70	879,1177	6,8	7,13	Yes
1.5625	128	839,60	829,10	876,716	5,4		1,10
		841,40	811,50	878,5956	7,6	6,53	Yes
3.125	64	864,10	829,60	902,2991	8,1		0,65
		855,00	809,20	892,7968	9,4	8,71	Yes
6.25	32	820,10	781,60	856,354	8,7		0,15
		856,30	813,40	894,1543	9,0	8,88	Yes
12.5	16	862,60	812,30	900,7328	9,8		0,31
		841,70	787,10	878,9088	10,4	10,13	Yes
25	8	824,50	769,10	860,9485	10,7		0,06
		861,60	802,60	899,6886	10,8	10,73	Yes
50	4	836,40	700,30	873,3746	19,8		0,28
		860,00	725,00	898,0178	19,3	19,54	Yes
100	2	876,20	598,10	914,934	34,6		0,09
		858,00	587,30	895,9294	34,4	34,54	Yes

Table 27a: Gamma values and gamma linear regression applied for EC-calculations in* V. fischeri *test.

Olefin-IMI

Conc. mg/l	% Inhibition	Gamma	log Gama Y	log conc. X	Gamma Lin. Reg	EC	Concentration mg/l
0,39	2,2549	0,02			0,02	20	22,577
0,78	2,1301	0,02			0,03	30	49,93
1,5625	6,7432	0,07			0,04	40	95,517
3,125	7,0641	0,08			0,07	50	173,217
6,25	10,9945	0,12	-0,908	0,796	0,10	60	314,351
12,50	12,4982	0,14	-0,845	1,097	0,17	70	601,678
25,00	20,3914	0,26	-0,592	1,398	0,27	80	1328,985
50,00	27,7358	0,38	-0,416	1,699	0,43		
100,00	44,3336	0,80	-0,099	2,00	0,69		

Desnitro-IMI

Conc. mg/l	% Inhibition	Gamma	log Gama Y	log conc. X	Gamma Lin. Reg	EC	Concentration mg/l
0,39	4,2739	0,04			0,0046	20	41,563
0,78	9,7416	0,11			0,01	30	78,125
1,5625	4,8058	0,05			0,02	40	130,856
3,125	4,963	0,05			0,03	50	210,059
6,25	5,7554	0,06			0,05	60	337,396
12,50	9,3826	0,10			0,09	70	565,362
25,00	14,9869	0,18	-0,754	1,398	0,16	80	1061,635
50,00	19,7913	0,25	-0,608	1,699	0,29		
100,00	36,599	0,58	-0,239	2,00	0,53		

5-hydroxy-IMI

Conc. mg/l	% Inhibition	Gamma	log Gama Y	log conc. X	Gamma Lin. Reg	EC	Concentration mg/l
0,39	6,0011	0,06			0,01	20	46,01
0,78	7,1255	0,08			0,01	30	92,821
1,5625	6,5339	0,07			0,02	40	164,721
3,125	8,7103	0,10			0,03	50	278,822
6,25	8,8803	0,10			0,05	60	472,265
12,50	10,1318	0,11	-0,948	1,097	0,09	70	838,478
25,00	10,7298	0,12	-0,92	1,398	0,16	80	1689,669
50,00	19,5417	0,24	-0,615	1,699	0,27		
100,00	34,5386	0,53	-0,278	2,00	0,45		

Table 28: Values of fluorescence measurement and % of algal growth in* D. subspicatus *exposed for 0, 24, 48, 72 and 96 h to IMI, Confidor 200SL and 6CNA.

D. subspicatus

0 h

Fluorescence measurments(FU)

mg L $^{-1}$	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*
positive control	431	371	387	387	408
	408	434	374	425	362
	375	383	374	376	338
control	363	388	376	391	401
	437	389	388	358	382
	404	414	379	201	428
imidacloprid	365	353	412	366	359
	399	406	397	387	359
	378	378	366	370	318
confidor 200SL	380	355	340	355	403
	400	373	350	352	390
	380	334	345	340	360
6CNA	405	330	332	368	277
	455	395	356	373	234
	444	402	395	393	254

Average

positive control *(mg L $^{-1}$)*	*0*	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*
Mean	379.933	404.667	396	378.333	396	369.333
Survival %	100	106.51	104.229	99.5789	104.229	97.21
SD	0	7.40878	8.80456	1.97549	6.76696	9.36257
imidacloprid						
Mean	379.933	380.667	379	391.667	374.333	345.333
Survival %	100	100.193	99.7543	103.088	98.5261	90.8931
SD	0	4.51557	6.97863	6.17455	2.93485	6.2304
confidor 200SL						
Mean	379.933	386.667	354	345	349	384.333
Survival %	100	101.772	93.1742	90.8054	91.8582	101.158
SD	0	3.03922	5.13754	1.31602	2.08912	5.80443
6CNA						
Mean	417.333	434.667	375.667	361	378	388.333
Survival %	100	104.153	90.016	86.5016	90.5751	93.0511
SD	0	6.29573	9.51351	7.6189	3.16983	9.86899

24 h

Fluorescence measurments(FU)

mg L $^{-1}$	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*
positive control	328	108	72	68	39
	521	131	71	95	29
	204	83	73	61	39
control	579	647	546	593	530
	761	587	649	557	620
	567	543	551	104	488
imidacloprid	465	435	427	548	445
	485	478	458	417	438
	478	433	439	456	387
confidor 200SL	647	541	400	415	418
	529	486	404	414	393
	513	435	412	375	358
6CNA	1060	1391	2055	2596	38
	896	962	1396	1800	41
	794	805	924	998	40

Average

positive control *(mg L $^{-1}$)*	*control*	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*
Mean	554.8	351	107.333	72	74.6667	35.6667
Survival %	100	63.266	19.3463	12.9776	13.4583	6.42874
SD	0	28.7936	4.32713	0.18025	3.23606	1.04065
imidacloprid						
Mean	554.8	476	448.667	441.333	473.667	423.333
Survival %	100	85.7967	80.87	79.5482	85.3761	76.3038
SD	0	1.82929	4.58239	2.81744	12.1239	5.7065
confidor 200SL						
Mean	554.8	563	487.333	405.333	401.333	389.667
Survival %	100	101.478	87.8395	73.0594	72.3384	70.2355
SD	0	13.1912	9.55526	1.10132	4.11154	5.43233
6CNA						
Mean	1517.13	916.667	1052.67	1458.33	1798	39.6667
Survival %	100	60.421	69.3852	96.1243	118.513	2.61458
SD	0	8.84555	19.9942	37.4437	52.6652	0.10068

Table 28: Values of fluorescence measurement and % of algal growth in* D. subspicatus *exposed for 0, 24, 48, 72 and 96 h to IMI, Confidor 200SL and 6CNA (continued).

D. subspicatus

48 h

Fluorescence measurments(FU)						
mg/L	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*	
positive control	173	35	24	19	13	
	892	52	30	21	9	
	109	35	23	18	8	
control	1234	1326	1317	1283	1084	
	1557	1401	1676	1320	1286	
	1273	1583	1413	468	1403	
imidacloprid	1665	1441	1408	1589	1481	
	1731	1797	1623	1495	1522	
	1710	1676	1661	1565	1652	
confidor 200SL	1919	1540	1244	1165	1137	
	1730	1550	1114	1090	1097	
	1836	1440	1145	959	981	
6CNA	3747	3659	5030	4487	20	
	4053	4669	5111	4173	15	
	4894	4118	5320	4365	20	

Average						
positive control *(mg L^{-1})*	*0*	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*
Mean	981.185	93.980	30.485	19.235	14.485	7.485
Survival %	100.000	9.578	3.107	1.960	1.476	0.763
SD	66.671	8.918	2.230	1.348	0.996	0.558
imidacloprid						
Mean	981.185	1,276.485	1,228.485	1,172.985	1,162.235	1,163.700
Survival %	100.000	130.096	125.204	119.548	118.452	118.605
SD	66.671	3.437	18.449	13.905	4.978	9.099
confidor 200SL						
Mean	981.185	1,371.235	1,132.485	875.735	803.485	803.735
Survival %	100.000	139.753	115.420	89.253	81.889	81.915
SD	66.671	9.655	6.199	6.920	10.626	8.258
6CNA						
Mean	2,920.133	4,231.333	4,148.667	5,153.667	4,341.667	18.333
Survival %	100.000	144.902	142.071	176.487	148.680	0.628
SD	0.000	20.339	17.318	5.124	5.421	0.099

72 h

Fluorescence measurments(FU)						
mg/L	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*	
positive control	153	24	17	14	13	
	1586	28	21	15	13	
	72	29	15	12	7	
control	4087	4524	4081	3834	3535	
	4255	3708	6175	3267	3307	
	3417	3702	3596	2120	3575	
imidacloprid	5338	4761	4148	4757	4277	
	5047	5329	5368	4503	4779	
	6404	7413	6117	5556	5624	
confidor 200SL	4991	3921	2621	2356	2018	
	5006	4214	2658	2198	1942	
	5497	4512	2907	2219	1844	
6CNA	5039	145	29	17	14	
	4055	357	39	11	14	
	6057	645	182	26	18	

Average						
positive control *(mg L^{-1})*	*control*	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*
Mean	3,812.200	112.500	27.000	17.667	13.667	11.000
Survival %	100.000	2.951	0.708	0.463	0.358	0.289
SD	0.000	1.502	0.069	0.080	0.040	0.091
imidacloprid						
Mean	3,812.200	5,596.333	5,834.333	5,211.000	4,938.667	4,893.333
Survival %	100.000	146.801	153.044	136.693	129.549	128.360
SD	0.000	18.741	36.628	26.070	14.414	17.857
confidor 200SL						
Mean	3,812.200	5,164.667	4,215.667	2,728.667	2,257.667	1,934.667
Survival %	100.000	135.477	110.584	71.577	59.222	50.749
SD	0.000	7.552	7.752	4.080	2.251	2.288
6CNA						
Mean	6,559.267	9,784.000	10,216.000	10,478.000	9,227.333	14.000
Survival %	100.000	149.163	155.749	159.743	140.676	0.213
SD	0.000	16.519	11.029	12.001	16.055	0.026

Table 28: Values of fluorescence measurement and % of algal growth in* D. subspicatus *exposed for 0, 24, 48, 72 and 96 h to IMI, Confidor 200SL and 6CNA (continued)

D. subspicatus

96 h

Fluorescence measurments(FU)						
mg/L	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*	
positive control	462	21	20	12	12	
	3866	21	21	18	15	
	203	27	20	10	9	
control	6471	6929	7019	7014	6887	
	7933	7510	9929	7681	7720	
	7864	8701	8095	6062	8140	
imidacloprid	9901	10912	10546	10565	8296	
	12089	12528	10834	9157	8494	
	11920	10684	10770	10443	8183	
confidor 200SL	10026	8694	6207	4321	3388	
	9711	9875	6780	5642	3576	
	9623	9501	7800	6471	4497	
6CNA	10620	10781	12295	10421	17	
	10452	12392	12239	9493	12	
	12070	11402	12322	10662	16	
Average						
positive control *(mg L $^{-1}$)*	*0*	*7.6*	*25.6*	*51.1*	*127.8*	*255.6*
Mean	7597	332.5	23	20.3333	13.3333	12
Survival %	100	4.37673	0.30275	0.26765	0.17551	0.15796
SD	0	2.4107	0.0456	0.0076	0.0548	0.03949
imidacloprid						
Mean	7597	11303.3	11374.7	10716.7	10055	8324.33
Survival %	100	148.787	149.726	141.064	132.355	109.574
SD	0	16.0246	13.2329	1.9906	10.2683	2.07219
confidor 200SL						
Mean	7597	9786.67	9356.67	6929	5478	3820.33
Survival %	100	128.823	123.163	91.2071	72.1074	50.2874
SD	0	2.7891	7.94504	10.6211	14.2733	7.81232
6CNA						
Mean	9526.73	11047.3	11525	12285.3	10192	15
Survival %	100	115.961	120.975	128.956	106.983	0.15745
SD	0	9.33825	8.52877	0.44439	6.47891	0.02777
DMSO + NMP						
% v/v	0	0.003	0.01	0.02	0.06	0.12
Mean	7597	6176.31	6054.8	4277.11	1344.66	774.8
Survival %	100	81.3	79.7	56.3	17.7	10.3
SD	0	4.56	6.23	7.32	3.21	6.02

Table 29:* G. fossarum *wet weight (g) and total body length (mm) measured during whole experimental trial. Dead individuals are signed by † and grey colour. n= 50 for every exposure concentration and tested compound IMI (A), Confidor 200SL (B) and 6CNA (C).

G. fossarum

A

control			**102.2 µg L^{-1}**			**153.3 µg L^{-1}**		
Wet weight		*Length*	*Wet weight*		*Length*	*Wet weight*		*Length*
0.017	†	**7**	***0.016***	†	***5***	***0.038***	†	***10***
0.026	†	**10**	***0.034***	†	***11***	***0.031***		***12***
0.017		9	***0.036***	†	***12***	***0.054***	†	***12***
0.038		12	***0.037***	†	***12***	***0.054***		***13***
0.052		14	***0.028***	†	***12***	***0.029***		***8***
0.052		15	***0.342***	†	***13***	***0.032***		***10***
0.054		15	***0.037***	†	***12***	***0.017***		***10***
0.036		19	***0.023***	†	***14***	0.043		16
0.018		16	0.018	†	10	0.032		14
0.044		14	0.068	†	14	0.027		13
0.019		10	0.026	†	9	0.017		11
0.032		12	0.018		9	0.023		11
0.032		10	0.053		16	0.054		12
0.038		7	0.053		15	0.03		11
0.043		14	0.024		12	0.061		16
0.031		10	0.027		12	0.049		12
0.052		14	0.054		14	0.031		12
0.052		15	0.046		13	0.054		13
0.046		17	0.067		14	0.029		8
0.024		10	0.052		10	0.045		17
0.047		15	0.08		16	0.043		16
0.023		11	0.023		10	0.032		15
0.026		11	0.018		10	0.038		16
0.035		13	0.056		17	0.037		13
0.041		15	0.029		11	0.038		11
0.023		11	0.029		11	0.023		12
0.03		14	0.023		11	0.026		12
0.044		15	0.017		11	0.024		12
0.031		13	0.069		16	0.031		13
0.029		12	0.058		16	0.028		12
0.022		10	0.034		14	0.022		10
0.032		15	0.037		13	0.036		15
0.047		16	0.037		14	0.021		12
0.039		14	0.013		10	0.024		12
0.023		11	0.045		15	0.022		12
0.03		14	0.034		11	0.025		12
0.019		11	0.036		13	0.04		15
0.023		12	0.033		14	0.038		12
0.043		16	0.018		12	0.038		16
0.038		15	0.026		13	0.032		12
0.04		17	0.028		14	0.028		14
0.027		12	0.045		16	0.03		14
0.041		15	0.038		14	0.047		15
0.024		11	0.022		14	0.028		10
0.028		13	0.027		15	0.04		15
0.032		13	0.038		16	0.038		11
0.036		13	0.037		16	0.027		13
0.032		17	0.032		16	0.043		14
0.043		17	0.022		10	0.032		12
0.042		15	0.043		17	0.042		15
0.032		14	0.041		15	0.034		11
0.036		11	0.032		13	0.024		13
0.023		12	0.021		12	0.016		13

Table 29:* G. fossarum *wet weight (g) and total body length (mm) measured during whole experimental trial. Dead individuals are signed by † and grey colour. n= 50 for every exposure concentration and tested compound IMI (A), Confidor 200SL (B) and 6CNA (C) (continued).

G. fossarum

A

204.5 µg L^{-1}			255.6 µg L^{-1}			511.3 µg L^{-1}		
Wet weight		*Length*	*Wet weight*		*Length*	*Wet weight*		*Length*
0.05	***†***	***13***	***0.023***	***†***	***6***	***0.038***	***†***	***15***
0.034	***†***	***11***	***0.025***	***†***	***6***	***0.03***	***†***	***11***
0.048	***†***	***15***	***0.033***	***†***	***11***	***0.032***	***†***	***15***
0.06	***†***	***17***	***0.07***	***†***	***17***	***0.038***	***†***	***14***
0.025	***†***	***12***	***0.041***	***†***	***15***	***0.032***	***†***	***15***
0.018	***†***	***11***	***0.033***	***†***	***16***	***0.032***	***†***	***15***
0.041	***†***	***15***	0.025		13	***0.038***	***†***	***15***
0.038	***†***	***15***	0.04		14	***0.245***		***11***
0.07		17	0.022		12	***0.038***		***14***
0.026		14	0.053		15	***0.034***		***15***
0.061		15	0.039		13	0.057		16
0.056		14	0.027		10	0.022		11
0.05		12	0.052		13	0.043		17
0.032		9	0.056		13	0.036		10
0.038		12	0.041		15	0.027		10
0.055		15	0.049		18	0.08		18
0.048		15	0.041		15	0.036		10
0.039		14	0.045		16	0.025		8
0.024		13	0.039		16	0.03		15
0.049		16	0.037		13	0.043		15
0.019		12	0.039		13	0.044		15
0.049		18	0.349		15	0.041		14
0.034		12	0.048		10	0.027		14
0.425		14	0.048		14	0.036		15
0.035		13	0.04		15	0.036		10
0.041		13	0.022		12	0.019		12
0.029		13	0.034		15	0.026		14
0.042		17	0.02		10	0.021		12
0.045		15	0.034		15	0.044		15
0.033		15	0.042		17	0.041		14
0.025		14	0.043		15	0.027		14
0.021		12	0.04		15	0.045		16
0.032		14	0.044		18	0.043		17
0.044		15	0.021		12	0.021		12
0.029		13	0.048		16	0.019		12
0.042		17	0.041		16	0.026		14
0.025		12	0.02		10	0.042		15
0.031		12	0.046		15	0.043		16
0.026		12	0.032		13	0.044		14
0.026		12	0.024		10	0.042		15
0.028		12	0.032		16	0.039		13
0.045		12	0.036		16	0.036		11
0.034		13	0.023		14	0.041		13
0.036		15	0.032		13	0.023		13
0.033		17	0.028		14	0.026		13
0.03		12	0.023		17	0.024		17
0.019		10	0.048		15	0.022		15
0.023		10	0.042		16	0.032		15
0.043		17	0.028		12	0.014		5
0.022		11	0.027		12	0.036		10
0.036		11	0.037		12	0.025		8
0.039		15	0.019		11	0.022		7

Table 29:* G. fossarum *wet weight (g) and total body length (mm) measured during whole experimental trial. Dead individuals are signed by † and grey colour. n= 50 for every exposure concentration and tested compound IMI (A), Confidor 200SL (B) and 6CNA (C) (continued).

G. fossarum

B

control / DMSO+ NMP			**102.2 µg L^{-1}**			**153.3 µg L^{-1}**
Wet weight		*Length*	*Wet weight*		*Length*	*Wet weight*
0.017	†	**7**	**0.019**	†	**7**	***0.015***
0.026	†	**10**	**0.026**	†	**10**	***0.024***
0.017		9	0.017		9	***0.038***
0.038		12	0.039		12	***0.037***
0.052		14	0.052		14	***0.029***
0.052		15	0.042		15	***0.342***
0.054		15	0.054		15	0.035
0.036		19	0.026		19	0.023
0.018		16	0.018		16	0.018
0.044		14	0.054		14	0.067
0.019		10	0.019		10	0.026
0.032		12	0.032		12	0.018
0.032		10	0.032		10	0.053
0.038		7	0.038		7	0.053
0.043		14	0.033		14	0.031
0.031		10	0.035		11	0.0542
0.052		14	0.04		14	0.0292
0.052		15	0.05		14	0.0318
0.046		17	0.036		17	0.0503
0.024		10	0.028		12	0.0345
0.047		15	0.047		15	0.08
0.023		11	0.013		10	0.023
0.026		11	0.026		11	0.018
0.035		13	0.035		12	0.056
0.041		15	0.041		15	0.029
0.023		11	0.033		11	0.029
0.03		14	0.03		13	0.038
0.044		15	0.044		13	0.03
0.031		13	0.021		13	0.032
0.029		12	0.029		12	0.038
0.022		10	0.022		10	0.032
0.032		15	0.032		15	0.032
0.047		16	0.047		16	0.038
0.039		14	0.039		14	0.013
0.023		11	0.023		11	0.045
0.03		14	0.03		14	0.034
0.019		11	0.027		10	0.034
0.023		12	0.052		14	0.033
0.043		16	0.056		13	0.028
0.038		15	0.041		15	0.02
0.04		17	0.039		18	0.028
0.027		12	0.041		15	0.042
0.041		15	0.046		14	0.038
0.024		11	0.024		11	0.025
0.028		13	0.028		13	0.027
0.032		13	0.035		13	0.039
0.036		13	0.036		13	0.037
0.032		17	0.032		15	0.033
0.043		17	0.023		13	0.042
0.042		15	0.042		15	0.043
0.032		14	0.042		14	0.031
0.036		11	0.036		11	0.032
0.023		12	0.023		12	0.021

Table 29:* G. fossarum *wet weight (g) and total body length (mm) measured during whole experimental trial. Dead individuals are signed by † and grey colour. n= 50 for every exposure concentration and tested compound IMI (A), Confidor 200SL (B) and 6CNA (C) (continued).

G. fossarum

B

204.5 µg L^{-1}			255.6 µg L^{-1}			511.3 µg L^{-1}		
Wet weight		*Length*	*Wet weight*		*Length*	*Wet weight*		*Length*
0.038	***†***	***10***	***0.023***	***†***	***6***	***0.023***	***†***	***6***
0.037	***†***	***12***	***0.025***	***†***	***6***	***0.025***	***†***	***6***
0.044	***†***	***12***	***0.033***	***†***	***11***	***0.033***	***†***	***12***
0.054	***†***	***13***	***0.07***	***†***	***17***	***0.072***	***†***	***15***
0.039	***†***	***8***	***0.019***	***†***	***7***	***0.044***	***†***	***15***
0.022	***†***	***10***	***0.026***	***†***	***10***	***0.033***	***†***	***16***
0.019	***†***	***10***	***0.017***	***†***	***9***	***0.026***	***†***	***14***
0.043	***†***	**16**	***0.039***	***†***	***12***	***0.04***	***†***	***15***
0.036	***†***	**14**	***0.016***	***†***	***5***	***0.023***	***†***	***12***
0.027	***†***	**13**	***0.034***	***†***	***11***	***0.053***	***†***	***15***
0.017	***†***	**11**	***0.036***	***†***	***12***	***0.036***	***†***	***13***
0.023		11	***0.037***	***†***	***12***	***0.019***	***†***	***11***
0.054		12	***0.038***	***†***	***10***	***0.028***	***†***	***12***
0.03		11	***0.031***	***†***	***12***	***0.043***	***†***	***16***
0.061		16	***0.054***	***†***	***12***	***0.038***	***†***	***15***
0.049		12	***0.054***	***†***	***13***	***0.04***	***†***	***17***
0.031		12	***0.0169***	***†***	***13***	***0.027***	***†***	***12***
0.054		13	***0.026***	***†***	***11***	***0.041***	***†***	***15***
0.029		8	***0.0173***	***†***	***15***	***0.014***	***†***	***5***
0.045		17	***0.038***	***†***	***17***	***0.036***	***†***	***10***
0.043		16	***0.037***	***†***	***12***	***0.025***	***†***	***8***
0.032		15	***0.023***	***†***	***14***	***0.022***	***†***	***7***
0.038		16	***0.018***	***†***	***10***	***0.042***	***†***	***15***
0.037		13	***0.068***	***†***	***14***	***0.039***	***†***	***13***
0.038		11	***0.026***	***†***	***9***	***0.036***	***†***	***11***
0.023		12	0.018		9	***0.041***	***†***	***13***
0.026		12	0.017		10	***0.034***	***†***	***15***
0.024		12	0.043		16	***0.026***	***†***	***10***
0.031		13	0.032		14	0.034		15
0.028		12	0.027		13	0.042		17
0.022		10	0.017		11	0.043		15
0.036		15	0.023		11	0.04		15
0.021		12	0.0541		15	0.044		18
0.024		12	0.0159		15	0.022		12
0.022		12	0.034		17	0.048		16
0.025		12	0.0362		14	0.041		16
0.04		15	0.018		15	0.02		10
0.038		12	0.0676		14	0.046		15
0.038		16	0.026		12	0.034		13
0.032		12	0.026		12	0.024		10
0.028		14	0.028		12	0.032		16
0.03		14	0.045		12	0.036		16
0.047		15	0.034		13	0.028		14
0.028		10	0.036		15	0.032		13
0.04		15	0.033		17	0.028		14
0.038		11	0.03		12	0.023		17
0.027		13	0.019		10	0.048		15
0.043		14	0.023		10	0.042		16
0.032		12	0.043		17	0.028		12
0.042		15	0.022		11	0.028		12
0.034		11	0.036		11	0.037		12
0.024		13	0.039		15	0.019		11
0.016		13	0.032		12	0.036		12

Table 29:* G. fossarum *wet weight (g) and total body length (mm) measured during whole experimental trial. Dead individuals are signed by † and grey colour. n= 50 for every exposure concentration and tested compound IMI (A), Confidor 200SL (B) and 6CNA (C) (continued).

G. fossarum

C

control			**102.2 µg L^{-1}**			**153.3 µg L^{-1}**		
Wet weight		*Length*	*Wet weight*		*Length*	*Wet weight*		*Length*
0.017	***†***	***7***	**0.08**	***†***	**18**	***0.017***	***†***	***8***
0.026	***†***	***10***	**0.036**	***†***	**10**	***0.023***	***†***	***12***
0.017		9	**0.025**	***†***	**8**	***0.038***	***†***	***13***
0.038		12	**0.03**	***†***	**15**	***0.037***	***†***	***12***
0.052		14	0.043		15	0.032		11
0.052		15	0.044		15	0.42		13
0.054		15	0.245		11	0.025		12
0.036		19	0.038		14	0.036		14
0.018		16	0.034		15	0.018		12
0.044		14	0.057		16	0.067		12
0.019		10	0.022		11	0.046		9
0.032		12	0.043		17	0.018		10
0.032		10	0.037		10	0.043		15
0.038		7	0.027		10	0.053		15
0.043		14	0.039		16	0.021		14
0.031		10	0.037		13	0.042		15
0.052		14	0.039		13	0.029		14
0.052		15	0.349		15	0.038		12
0.046		17	0.048		10	0.03		16
0.024		10	0.048		14	0.035		11
0.047		15	0.04		15	0.08		16
0.023		11	0.041		14	0.02		10
0.026		11	0.027		14	0.018		10
0.035		13	0.036		15	0.056		17
0.041		15	0.036		10	0.039		11
0.023		11	0.019		12	0.029		12
0.03		14	0.026		14	0.018		15
0.044		15	0.021		12	0.03		11
0.031		13	0.044		15	0.032		13
0.029		12	0.041		14	0.028		12
0.022		10	0.027		14	0.032		15
0.032		15	0.045		16	0.032		15
0.047		16	0.043		17	0.018		14
0.039		14	0.021		12	0.023		10
0.023		11	0.01		12	0.045		15
0.03		14	0.026		14	0.034		11
0.019		11	0.042		15	0.034		13
0.023		12	0.043		16	0.033		14
0.043		16	0.044		14	0.028		12
0.038		15	0.042		15	0.024		13
0.04		17	0.039		13	0.028		14
0.027		12	0.036		11	0.042		16
0.041		15	0.041		13	0.038		14
0.024		11	0.023		13	0.025		14
0.028		13	0.026		13	0.017		13
0.032		13	0.024		17	0.039		14
0.036		13	0.022		15	0.037		16
0.032		17	0.032		15	0.033		16
0.043		17	0.032		17	0.042		17
0.042		15	0.036		15	0.053		17
0.032		14	0.046		16	0.031		15
0.036		11	0.032		12	0.022		13
0.023		12	0.041		16	0.021		11

Table 29:* G. fossarum *wet weight (g) and total body length (mm) measured during whole experimental trial. Dead individuals are signed by † and grey colour. n= 50 for every exposure concentration and tested compound IMI (A), Confidor 200SL (B) and 6CNA (C) (continued).

G. fossarum

C

204.5 µg L^{-1}			255.6 µg L^{-1}			511.3 µg L^{-1}		
Wet weight		*Length*	*Wet weight*		*Length*	*Wet weight*		*Length*
0.032	***†***	***10***	***0.038***	***†***	***15***	***0.0142***	***†***	***10***
0.038	***†***	***7***	***0.036***	***†***	***10***	***0.0193***	***†***	***11***
0.043	***†***	***14***	***0.027***	***†***	***10***	***0.201***	***†***	***18***
0.031	***†***	***10***	***0.080***	***†***	***18***	***0.072***	***†***	***14***
0.052	***†***	***14***	***0.036***	***†***	***10***	***0.044***	***†***	***18***
0.052		15	***0.025***	***†***	***8***	***0.033***	***†***	***12***
0.046		17	0.030		15	***0.026***	***†***	***17***
0.024		10	0.043		15	0.04		12
0.047		15	0.044		15	0.023		12
0.023		11	0.041		14	0.053		12
0.026		11	0.027		14	0.036		16
0.067		14	0.0218		12	0.019		8
0.052		10	0.0208		10	0.028		
0.023		16	0.0423		12	0.043		19
0.023		10	0.0236		12	0.038		13
0.018		10	0.0343		13	0.04		15
0.056		17	0.0198		13	0.027		12
0.029		11	0.0383		11	0.041		10
0.029		11	0.0486		15	0.014		10
0.023		11	0.0213		17	0.036		15
0.017		11	0.0423		12	0.025		15
0.054		12	0.0394		14	0.022		12
0.061		11	0.032		10	0.042		11
0.061		16	0.0423		14	0.039		18
0.049		12	0.0432		9	0.036		14
0.031		12	0.0283		9	0.041		17
0.054		13	0.0301		10	0.034		13
0.029		8	0.0238		16	0.026		10
0.045		17	0.0324		14	0.034		14
0.043		16	0.0183		13	0.042		11
0.032		15	0.0239		11	0.043		11
0.038		16	0.0313		11	0.04		11
0.037		13	0.0193		15	0.044		11
0.031		12	0.0182		15	0.022		14
0.032		9	0.0473		17	0.048		16
0.038		12	0.0306		14	0.041		17
0.055		15	0.0412		15	0.02		18
0.048		15	0.0273		14	0.046		16
0.039		14	0.0201		12	0.034		11
0.024		13	0.0423		12	0.024		11
0.049		16	0.02		12	0.032		17
0.019		12	0.0197		12	0.036		14
0.049		18	0.0132		13	0.028		11
0.039		13	0.0214		15	0.032		13
0.027		10	0.0227		17	0.028		14
0.052		13	0.0412		12	0.023		17
0.056		13	0.0432		10	0.048		15
0.041		15	0.0327		10	0.042		16
0.049		18	0.0402		17	0.028		12
0.041		15	0.0187		11	0.028		12
0.045		16	0.0156		11	0.037		12
0.039		16	0.0428		15	0.019		11
0.037		13	0.0231		12	0.036		12

Table 30: CAT activity in* G. fossarum *exposed to IMI (A), Confidor 200SL (B) and 6CNA (C) for 24 h. Representative absorbance (AU) values of whole 2 min measurement (selected for every 30 s) and data normalised on total protein content (µmol/min/mg protein). (n=10).

CAT

24 h - A

Control										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.249984	0.236015	0.328127	0.189747	0.944999	0.526705	0.15148	0.103817	0.147454	0.26248
30 s	0.1434	0.144931	0.267479	0.124783	0.884891	0.39884	0.076513	0.041044	0.071584	0.16636
60 s	0.1278	0.107958	0.261221	0.110829	0.878326	0.449446	0.040124	0.003254	0.034201	0.13993
90 s	0.1225	0.095192	0.261815	0.109345	0.876684	0.466036	0.025223	0.014785	0.016996	0.13353
120 s	0.1319	0.092474	0.262044	0.10956	0.872544	0.461109	0.024692	0.020759	0.00724	0.13065
102.2 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.622296	0.378397	0.248703	0.244712	0.562342	0.303023	0.134211	0.29079	0.148624	0.43806
30 s	0.510951	0.309595	0.192585	0.17747	0.51609	0.205498	0.081636	0.211563	0.073619	0.09428
60 s	0.473377	0.300228	0.186722	0.156488	0.513651	0.194052	0.044354	0.186296	0.044421	0.08821
90 s	0.4616	0.299063	0.189618	0.15161	0.513624	0.197784	0.023377	0.179639	0.030781	0.08333
120 s	0.459534	0.298819	0.193574	0.150546	0.512424	0.207905	0.015575	0.178033	0.019442	0.07377
153.3 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.387351	0.293125	0.232242	0.368594	0.208142	0.21532	0.421714	0.199429	0.099711	0.3391
30 s	0.321101	0.228271	0.156464	0.331384	0.123799	0.145265	0.297398	0.136172	0.032089	0.2359
60 s	0.316213	0.223401	0.135936	0.329843	0.09466	0.102403	0.275566	0.119574	0.007261	0.23418
90 s	0.317878	0.223038	0.129975	0.331618	0.088608	0.079409	0.271171	0.115876	0.028495	0.23091
120 s	0.3209	0.223233	0.127897	0.332949	0.094649	0.067767	0.273193	0.115107	0.040036	0.20964
204.5 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.109651	0.568216	0.308946	0.456586	0.51346	0.384129	0.196056	0.157861	0.218183	0.39877
30 s	0.107902	0.48655	0.254737	0.415276	0.465719	0.311387	0.106084	0.087623	0.147254	0.28689
60 s	0.10576	0.447169	0.245754	0.414603	0.461604	0.30384	0.091746	0.05773	0.096521	0.30647
90 s	0.105092	0.427466	0.244844	0.414191	0.461798	0.307386	0.098287	0.04877	0.068282	0.3165
120 s	0.104304	0.417472	0.245152	0.413845	0.462283	0.312876	0.109563	0.047428	0.054617	0.32143
255.6 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.370032	0.222188	0.308351	0.72374	0.265941	0.122743	0.189076	0.434314	0.370469	0.67924
30 s	0.2753	0.181938	0.230039	0.708714	0.172579	0.044143	0.12084	0.352311	0.285773	0.60047
60 s	0.23435	0.170581	0.216697	0.722104	0.144939	0.017542	0.077435	0.321198	0.249752	0.61264
90 s	0.221666	0.17146	0.215311	0.72614	0.137805	0.011349	0.055976	0.31635	0.240864	0.60824
120 s	0.220359	0.176104	0.217619	0.734509	0.138946	0.012736	0.047281	0.31852	0.239757	0.59708
511.3 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.372761	0.257039	0.295672	0.214039	0.207367	0.414632	0.359254	0.220962	0.206582	0.28352
30 s	0.318241	0.170586	0.225766	0.214832	0.152519	0.392458	0.255978	0.145373	0.123168	0.21291
60 s	0.280295	0.124015	0.214258	0.215311	0.142815	0.389164	0.244308	0.148769	0.10143	0.19975
90 s	0.265205	0.106573	0.213684	0.216409	0.142374	0.39124	0.250666	0.162347	0.094843	0.20069
120 s	0.258302	0.103872	0.212975	0.21806	0.142141	0.396799	0.260805	0.177832	0.095391	0.20278

Calculated values normalised on protein content

24 h	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**
control	3.30	0.14	23.26	3.61	0.17	21.04	2.44	0.14	17.20
102.2 µg L^{-1}	4.27	0.17	25.11	3.42	0.17	20.62	/	/	/
153.3 µg L^{-1}	2.93	0.15	20.21	3.26	0.16	21.04	4.68	0.17	28.20
204.5 µg L^{-1}	3.23	0.14	23.26	3.57	0.17	21.04	3.15	0.16	20.34
255.6 µg L^{-1}	3.92	0.13	29.92	4.06	0.12	34.78	12.01	0.13	90.31
511.3 µg L^{-1}	3.00	0.12	25.64	1.71	0.15	11.56	4.54	0.17	26.38
control	3.34	0.14	23.58	3.67	0.14	25.90	2.77	0.17	16.70
102.2 µg L^{-1}	2.08	0.13	16.64	6.75	0.13	54.06	/	/	/
153.3 µg L^{-1}	2.99	0.13	23.58	2.30	0.17	13.56	2.01	0.12	17.20
204.5 µg L^{-1}	3.62	0.17	21.34	3.68	0.15	25.40	2.55	0.15	17.20
255.6 µg L^{-1}	1.12	0.17	6.51	4.49	0.14	32.34	/	/	/
511.3 µg L^{-1}	4.02	0.15	27.16	3.54	0.15	23.94	2.09	0.13	15.58
control	2.34	0.16	14.30	3.65	0.16	22.30			
102.2 µg L^{-1}	1.45	0.16	9.04	3.61	0.16	22.59			
153.3 µg L^{-1}	3.89	0.16	24.35	4.68	0.21	22.30			
204.5 µg L^{-1}	2.03	0.14	14.30	3.79	0.17	22.30			
255.6 µg L^{-1}	1.21	0.13	9.22	3.91	0.14	27.55			
511.3 µg L^{-1}	2.38	0.17	14.00	3.30	0.13	25.18			
control	4.41	0.16	27.96	4.68	0.17	28.20			
102.2 µg L^{-1}	1.31	0.17	7.70	4.82	0.16	31.10			
153.3 µg L^{-1}	4.61	0.17	27.96	2.22	0.13	16.70			
204.5 µg L^{-1}	2.96	0.17	17.89	2.34	0.14	16.70			
255.6 µg L^{-1}	0.47	0.16	3.01	3.50	0.16	22.44			
511.3 µg L^{-1}	2.17	0.13	16.31	2.61	0.16	16.31			

Table 30: CAT activity in* G. fossarum *exposed to IMI (A), Confidor 200SL (B) and 6CNA (C) for 24 h. Representative absorbance (AU) values of whole 2 min measurement (selected for every 30 s) and data normalised on total protein content (µmol/min/mg protein). (n=10), (continued).

CAT

24 h - B

Control

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.249984	0.236015	0.328127	0.189747	0.944999	0.526705	0.15148	0.103817	0.147454	0.26248
30 s	0.1434	0.144931	0.267479	0.124783	0.884891	0.39884	0.076513	0.041044	0.071584	0.16636
60 s	0.1278	0.107958	0.261221	0.110829	0.878326	0.449446	0.040124	0.003254	0.034201	0.13993
90 s	0.1225	0.095192	0.261815	0.109345	0.876684	0.466036	0.025223	0.014785	0.016996	0.13353
120 s	0.1319	0.092474	0.262044	0.10956	0.872544	0.461109	0.024692	0.020759	0.00724	0.13065

102.2 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.522296	0.38397	0.248703	0.23414	0.5232	0.289814197	0.29285006	0.26924486	0.278629182	0.25714
30 s	0.510951	0.38595	0.198534	0.18726	0.52341	0.222244718	0.25269492	0.193485563	0.261264094	0.23475
60 s	0.473377	0.300228	0.185623	0.16285	0.51321	0.156715849	0.24618021	0.173053472	0.240855059	0.19025
90 s	0.4616	0.29963	0.18431	0.15064	0.51201	0.106401488	0.23923729	0.161487643	0.198077666	0.17877
120 s	0.359534	0.29819	0.183574	0.150741	0.512	0.100619267	0.21348412	0.132165169	0.182304731	0.17474

153.3 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.158624	0.43763	0.295672	0.214039	0.196056	0.21532	0.421714	0.199429	0.099711	0.3391
30 s	0.093619	0.2341	0.225766	0.214832	0.106084	0.145265	0.297398	0.136172	0.032089	0.2359
60 s	0.054431	0.08549	0.214258	0.215311	0.091746	0.102403	0.275566	0.119574	0.007261	0.23418
90 s	0.023783	0.08235	0.213684	0.216409	0.098287	0.079409	0.271171	0.115876	0.028495	0.23091
120 s	0.01946	0.07323	0.212975	0.21806	0.109563	0.067767	0.273193	0.115107	0.040036	0.20964

204.5 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.109651	0.568216	0.308946	0.456586	0.51346	0.385123	0.232121	0.157861	0.218183	0.39877
30 s	0.107902	0.48655	0.254737	0.415276	0.465719	0.311341	0.146464	0.087421	0.147254	0.28689
60 s	0.10576	0.447169	0.245754	0.414603	0.461604	0.31352	0.135923	0.04772	0.096521	0.30647
90 s	0.105092	0.427466	0.244844	0.414191	0.461798	0.30723	0.129295	0.041237	0.068282	0.3165
120 s	0.104304	0.417472	0.245152	0.413845	0.462283	0.30235	0.127897	0.037428	0.054617	0.32143

255.6 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.37123	0.22215	0.307653	0.72374	0.206582	0.189076	0.265941	0.434314	0.122743	0.67924
30 s	0.2681	0.2231	0.220456	0.708714	0.124566	0.12084	0.172579	0.352311	0.044143	0.60142
60 s	0.22319	0.222	0.21035	0.722104	0.100233	0.077435	0.15679	0.321198	0.017542	0.60344
90 s	0.2241	0.20345	0.20458	0.72614	0.090345	0.055976	0.137805	0.31635	0.011349	0.63454
120 s	0.22012	0.1934	0.200134	0.734509	0.08391	0.047281	0.124546	0.31852	0.012736	0.59756

511.3 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.372761	0.257039	0.368594	0.208142	0.207367	0.414632	0.359254	0.220962	0.290469	0.28352
30 s	0.35451	0.170586	0.331384	0.123799	0.152519	0.392458	0.255978	0.175372	0.284573	0.21291
60 s	0.28195	0.124015	0.329843	0.09466	0.142815	0.389164	0.24138	0.148769	0.26527	0.19975
90 s	0.27532	0.11173	0.321517	0.088608	0.142374	0.39124	0.25661	0.152347	0.24459	0.20069
120 s	0.258321	0.10342	0.302149	0.094649	0.142141	0.396799	0.24305	0.13256	0.23977	0.20278

Calculated values normalised on protein content

24 h	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**
control	3.30	0.14	23.26	3.61	0.17	21.04	2.44	0.14	17.20
102.2 µg L^{-1}	1.54	0.07	21.12	3.59	0.26	13.71	2.64	0.23	11.42
153.3 µg L^{-1}	1.41	0.07	20.21	5.28	0.25	21.04	7.08	0.25	28.20
204.5 µg L^{-1}	1.61	0.08	20.21	3.33	0.24	14.00	7.51	0.28	27.00
255.6 µg L^{-1}	3.00	0.08	39.73	2.86	0.26	10.95	6.83	0.23	29.18
511.3 µg L^{-1}	0.42	0.05	78.08	2.56	0.19	13.33	12.19	0.17	72.14
control	3.34	0.14	23.58	3.67	0.14	25.90	2.77	0.17	16.70
102.2 µg L^{-1}	1.83	0.05	35.26	3.16	0.12	26.55	/	/	/
153.3 µg L^{-1}	0.69	0.05	13.58	3.70	0.16	23.56	3.66	0.21	17.20
204.5 µg L^{-1}	1.06	0.05	23.58	2.28	0.17	13.56	3.54	0.18	19.23
255.6 µg L^{-1}	3.67	0.03	123.56	2.86	0.27	10.00	4.57	0.17	27.23
511.3 µg L^{-1}	2.53	0.03	78.08	19.09	0.27	71.23	14.28	0.18	78.02
control	2.34	0.16	14.30	3.65	0.16	22.30			
102.2 µg L^{-1}	1.51	0.16	9.20	3.47	0.29	12.13			
153.3 µg L^{-1}	4.01	0.17	24.35	5.46	0.25	22.30			
204.5 µg L^{-1}	4.09	0.17	24.35	4.94	0.25	20.00			
255.6 µg L^{-1}	3.40	0.14	23.61	3.11	0.12	25.70			
511.3 µg L^{-1}	2.89	0.27	10.58	5.48	0.12	45.26			
control	4.41	0.16	27.96	4.68	0.17	28.20			
102.2 µg L^{-1}	0.75	0.16	4.65	3.03	0.15	20.89			
153.3 µg L^{-1}	4.08	0.23	17.45	3.91	0.15	26.81			
204.5 µg L^{-1}	5.98	0.21	27.96	2.47	0.15	16.70			
255.6 µg L^{-1}	2.13	0.28	7.60	2.44	0.14	17.55			
511.3 µg L^{-1}	1.57	0.27	5.88	3.90	0.14	28.03			

Table 30: CAT activity in* G. fossarum *exposed to IMI (A), Confidor 200SL (B) and 6CNA (C) for 24 h. Representative absorbance (AU) values of whole 2 min measurement (selected for every 30 s) and data normalised on total protein content (µmol/min/mg protein). (n=10), (continued).

CAT

24 h - C

Control

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.417296	0.223984	0.447792	0.280212	0.282202	0.308197	0.15148	0.103817	0.156924	0.26248
30 s	0.289438	0.113222	0.295567	0.16861	0.153354	0.206356	0.08513	0.041044	0.091584	0.18635
60 s	0.301048	0.084728	0.382694	0.11518	0.151725	0.18544	0.04124	0.003654	0.0458201	0.13993
90 s	0.307308	0.072444	0.370074	0.104115	0.144534	0.154564	0.035223	0.014995	0.026396	0.1329
120 s	0.304934	0.071731	0.379914	0.100404	0.132029	0.137677	0.024539	0.020759	0.00921	0.1361

102.2 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.280964	0.469795	0.300103	0.406851	0.478454	0.51487	0.2928501	0.26243884	0.278684764	0.25894
30 s	0.168339	0.351609	0.150794	0.243353	0.318117	0.381618	0.2578167	0.19411345	0.26004986	0.2322
60 s	0.164014	0.378154	0.113745	0.260187	0.339945	0.382504	0.2474515	0.17630525	0.24079709	0.19238
90 s	0.175256	0.371781	0.124816	0.254015	0.344779	0.400398	0.2186981	0.16448088	0.1927135	0.17532
120 s	0.177516	0.371946	0.131744	0.243844	0.333275	0.391553	0.2170096	0.13275557	0.18059799	0.17627

153.3 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.15435	0.43763	0.295472	0.214039	0.195969	0.568216	0.308946	0.199429	0.385123	0.23537
30 s	0.09619	0.233	0.24591	0.21662	0.16943	0.48655	0.254737	0.147011	0.323971	0.14493
60 s	0.056793	0.08549	0.214958	0.215311	0.09946	0.447169	0.245754	0.12395	0.314891	0.14393
90 s	0.023569	0.08435	0.219714	0.216409	0.09596	0.427466	0.244844	0.115921	0.30491	0.14885
120 s	0.019502	0.07458	0.210365	0.215936	0.0235	0.417472	0.245152	0.115592	0.30855	0.1279

204.5 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.104551	0.21532	0.421714	0.456586	0.51346	0.099711	0.339102	0.157861	0.218183	0.39877
30 s	0.104402	0.1456765	0.2934498	0.415276	0.465719	0.032089	0.33593	0.087326	0.147254	0.29989
60 s	0.103	0.10456	0.211166	0.414603	0.461604	0.02261	0.3341	0.04767	0.106521	0.30047
90 s	0.103092	0.0759	0.2111	0.414191	0.461798	0.021495	0.280905	0.0414555	0.098282	0.3165
120 s	0.102304	0.0719356	0.20333	0.413845	0.462283	0.020035	0.59687	0.038923	0.084617	0.31114

255.6 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.695786	0.190596	0.482663	0.231408	0.170252	0.1896986	0.265941	0.434314	0.122743	0.35025
30 s	0.578729	0.05812	0.352929	0.073957	0.05696	0.167924	0.252579	0.352311	0.044143	0.27892
60 s	0.582328	0.015624	0.355879	0.030101	0.013514	0.156435	0.25679	0.321198	0.017542	0.24346
90 s	0.588393	0.016179	0.357232	0.008665	0.016508	0.1545976	0.237815	0.31635	0.011349	0.23166
120 s	0.595026	0.034017	0.347654	0.016339	0.032874	0.147281	0.244546	0.31852	0.012736	0.22231

511.3 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
0 s	0.292449	0.202655	0.294565	0.175453	0.500334	0.190094	0.679242	0.220962	0.290469	0.28352
30 s	0.158771	0.088393	0.17603	0.074383	0.353917	0.093607	0.60142	0.175372	0.284563	0.27603
60 s	0.120821	0.040104	0.141868	0.028976	0.394518	0.060739	0.60344	0.148769	0.278902	0.24507
90 s	0.10851	0.00626	0.118905	0.005186	0.397787	0.041749	0.634543	0.152347	0.265459	0.20694
120 s	0.093683	0.020868	0.112824	0.029738	0.397524	0.028976	0.59756	0.13256	0.2567977	0.20659

Calculated values normalised on protein content

24 h	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**	**µmol/min**	**mg protein sample**	**µmol/min/mg prot**
control	3.30	0.14	23.26	3.61	0.17	21.04	2.44	0.14	17.20
102.2 µg L^{-1}	2.71	0.12	22.17	3.81	0.13	29.17	4.86	0.15	31.56
153.3 µg L^{-1}	4.53	0.15	30.22	3.71	0.17	22.50	3.62	0.12	29.45
204.5 µg L^{-1}	2.65	0.26	10.23	3.37	0.16	20.79	4.21	0.14	29.65
255.6 µg L^{-1}	2.64	0.13	20.98	8.22	0.10	85.09	5.03	0.12	41.20
511.3 µg L^{-1}	5.21	0.11	46.10	2.69	0.10	26.40	8.72	0.14	62.30
control	3.34	0.14	23.58	3.67	0.14	25.90	2.77	0.17	16.70
102.2 µg L^{-1}	2.56	0.11	23.44	3.23	0.14	23.56	4.22	0.18	23.20
153.3 µg L^{-1}	2.64	0.11	23.58	4.87	0.15	33.56	2.87	0.15	19.23
204.5 µg L^{-1}	1.61	0.12	13.45	1.51	0.10	14.56	3.56	0.15	24.56
255.6 µg L^{-1}	9.19	0.14	67.30	10.01	0.15	69.03	4.80	0.12	40.00
511.3 µg L^{-1}	6.83	0.11	61.81	4.22	0.13	33.71	4.55	0.12	37.89
control	2.34	0.16	14.30	3.65	0.16	22.30			
102.2 µg L^{-1}	4.41	0.11	39.17	3.68	0.17	22.31			
153.3 µg L^{-1}	1.38	0.11	12.57	3.26	0.17	19.78			
204.5 µg L^{-1}	3.04	0.12	24.53	3.76	0.17	22.13			
255.6 µg L^{-1}	3.54	0.10	35.79	4.13	0.16	25.34			
511.3 µg L^{-1}	4.77	0.11	44.33	7.49	0.16	45.67			
control	4.41	0.16	27.96	4.68	0.17	28.20			
102.2 µg L^{-1}	4.27	0.13	32.57	4.07	0.17	23.40			
153.3 µg L^{-1}	4.05	0.15	27.56	2.76	0.17	16.70			
204.5 µg L^{-1}	4.71	0.15	32.45	5.87	0.17	35.60			
255.6 µg L^{-1}	7.79	0.20	38.97	10.32	0.16	65.34			
511.3 µg L^{-1}	7.69	0.10	74.44	7.27	0.16	44.30			

Table 31: GST activity in* G. fossarum *exposed to IMI (A), Confidor 200SL (B) and 6CNA (C) for 24 h. Representative absorbance (AU) values of whole 5 min measurement (selected for every min) and data normalised on total protein content (nmol/min/mg protein). (n=10).

GST

24 h - A

Control

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.146236	0.042268	0.118228	-0.025388	0.238809	0.225794	0.268316	0.37774	0.256141	0.13408
2 min	0.215348	0.092	0.180029	0.015482	0.32088	0.265945	0.313015	0.462245	0.24998	0.21085
3 min	0.277997	0.13941	0.234261	0.054798	0.348072	0.387399	0.42122	0.518991	0.249274	0.33364
4 min	0.333979	0.179978	0.279339	0.102651	0.3573	0.409343	0.4467295	0.563051	0.362658	0.38016
5 min	0.384914	0.21773	0.322515	0.129369	0.457125	0.444866	0.424734	0.593668	0.377037	0.40418

102.2 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.909622	0.802723	1.176711	1.074273	0.222303	0.460296	0.39944	0.44331	0.220164	0.14542
2 min	1.545629	0.933118	1.428991	1.243378	0.388799	0.79716	0.656945	0.709702	0.381393	0.28287
3 min	2.144172	1.053127	1.653984	1.39861	0.541888	1.093051	0.888451	0.948591	0.529834	0.41317
4 min	2.7892	1.16699	1.859892	1.546903	0.687614	1.357828	1.099222	1.166439	0.669636	0.53381
5 min	3.45683	1.275071	2.048848	1.687243	0.825741	1.597588	1.289486	1.363909	0.801064	0.64913

153.3 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.590015	1.021672	1.132618	0.469923	0.366457	0.124729	0.186352	0.056928	0.102605	0.17689
2 min	0.999548	1.188764	1.289452	0.769374	0.615384	0.229973	0.350374	0.220762	0.221323	0.19082
3 min	1.357925	1.34323	1.436688	1.039295	0.838152	0.32992	0.500128	0.373464	0.332224	0.20525
4 min	1.696015	1.480263	1.573813	1.282897	1.001446	0.423767	0.641188	0.516052	0.437991	0.21928
5 min	2.02275	1.612571	1.703852	1.503956	1.170568	0.514482	0.770873	0.650197	0.538277	0.23116

204.5 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.817308	1.056696	1.131106	0.280531	0.348402	0.19448	0.054815	0.115823	0.359723	0.98658
2 min	1.355639	1.277275	1.313312	0.496001	0.566677	0.35748	0.217042	0.207232	0.496969	1.07743
3 min	1.829354	1.481491	1.48226	0.693072	0.766985	0.506428	0.365746	0.293624	0.623925	1.16675
4 min	2.273806	1.666513	1.638484	0.876962	0.953984	0.647927	0.503935	0.376172	0.743778	1.25782
5 min	2.895171	1.832829	1.785761	1.049342	1.129494	0.779441	0.632618	0.454382	0.856411	1.34941

255.6 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.973248	1.046696	0.870266	0.140116	0.376066	0.399981	-0.058228	-0.002271	0.064088	0.37052
2 min	1.487858	1.191279	0.888636	0.284721	0.651402	0.659692	0.064438	0.130072	0.132022	0.54618
3 min	1.850204	1.327096	0.906303	0.419976	0.901567	0.897886	0.179641	0.257808	0.197218	0.71663
4 min	2.104055	1.452359	0.92442	0.546363	1.128708	1.132115	0.28668	0.381754	0.257507	0.87727
5 min	2.368362	1.574003	0.942526	0.666827	1.342134	1.346819	0.388663	0.501742	0.31682	1.04344

511.3 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	1.122983	0.272581	1.107798	1.004165	0.115112	0.398807	0.798974	0.274961	0.366106	0.04136
2 min	1.746232	0.310429	1.286498	1.130215	0.243017	0.678667	1.262047	0.444148	0.609996	0.09187
3 min	2.158413	0.346949	1.45279	1.249372	0.36618	0.926952	1.662634	0.601651	0.835636	0.14179
4 min	2.35678	0.385358	1.608845	1.363838	0.48214	1.152651	2.005436	0.746919	1.044832	0.18935
5 min	2.459443	0.420955	1.75555	1.478732	0.591267	1.361604	2.332274	0.883645	1.240717	0.23819

Calculated values normalised on protein content

24 h	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**
control	0.07	0.08	833	0.01	0.06	157	0.01	0.06	156
102.2 µg L^{-1}	0.12	0.09	1410	0.05	0.07	696	/	0.09	/
153.3 µg L^{-1}	0.05	0.06	833	0.04	0.08	457	0.02	0.09	201
204.5 µg L^{-1}	0.02	0.07	320	0.06	0.08	734	0.07	0.12	621
255.6 µg L^{-1}	0.05	0.08	650	0.07	0.08	878	0.14	0.14	978
511.3 µg L^{-1}	0.06	0.08	789	0.05	0.10	474	0.02	0.12	133
control	0.09	0.08	1158	0.02	0.07	261	0.03	0.08	324
102.2 µg L^{-1}	0.05	0.08	600	0.03	0.08	369	/	0.08	/
153.3 µg L^{-1}	0.03	0.06	458	0.04	0.08	464	0.01	0.06	224
204.5 µg L^{-1}	0.03	0.08	340	0.02	0.06	359	0.03	0.11	234
255.6 µg L^{-1}	0.04	0.08	480	0.03	0.05	632	0.04	0.11	326
511.3 µg L^{-1}	0.06	0.09	635	0.02	0.03	506	/	0.12	/
control	0.03	0.07	380	0.03	0.10	313			
102.2 µg L^{-1}	0.02	0.06	435	0.02	0.07	269			
153.3 µg L^{-1}	0.03	0.08	380	0.04	0.07	513			
204.5 µg L^{-1}	0.02	0.08	245	0.07	0.13	504			
255.6 µg L^{-1}	0.03	0.07	518	0.06	0.09	623			
511.3 µg L^{-1}	0.03	0.07	466	0.12	0.15	827			
control	0.03	0.08	351	0.03	0.11	258			
102.2 µg L^{-1}	0.04	0.07	564	0.04	0.07	597			
153.3 µg L^{-1}	0.03	0.06	551	0.02	0.06	258			
204.5 µg L^{-1}	0.05	0.09	562	0.03	0.12	302			
255.6 µg L^{-1}	0.04	0.10	383	0.03	0.08	393			
511.3 µg L^{-1}	0.10	0.12	795	0.02	0.12	146			

Table 31: GST activity in* G. fossarum *exposed to IMI (A), Confidor 200SL (B) and 6CNA (C) for 24 h. Representative absorbance (AU) values of whole 5 min measurement (selected for every min) and data normalised on total protein content (nmol/min/mg protein). (n=10), (continued).

GST

24 h - B

Control										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.146236	0.042268	0.118228	-0.025388	0.238809	0.225794	0.268316	0.37774	0.256141	0.13408
2 min	0.215348	0.092	0.180029	0.015482	0.32088	0.265945	0.313015	0.462245	0.24998	0.21085
3 min	0.277997	0.13941	0.234261	0.054798	0.348072	0.387399	0.42122	0.518991	0.249274	0.33364
4 min	0.333979	0.179978	0.279339	0.102651	0.3573	0.409343	0.4467295	0.563051	0.362658	0.38016
5 min	0.384914	0.21773	0.322515	0.129369	0.457125	0.444866	0.424734	0.593668	0.377037	0.40418
102.2 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.246487	0.214286	0.165125	0.1922	0.231688	0.099655	0.205051	0.20567	0.16857	0.34794
2 min	0.463121	0.412305	0.322555	0.32956	0.40615	0.209559	0.373268	0.3568	0.356932	0.47834
3 min	0.65974	0.595308	0.467042	0.478132	0.569532	0.314147	0.531424	0.49535	0.46854	0.7792
4 min	0.842501	0.764909	0.594008	0.576049	0.725613	0.416258	0.6795	0.793	0.57891	0.98673
5 min	1.011514	0.926191	0.707406	0.723529	0.876813	0.512416	0.836608	0.98214	0.61234	1.05743
153.3 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.278601	0.342678	0.411578	0.977062	1.045793	0.209089	0.213854	0.39062	0.164019	0.20859
2 min	0.498475	0.593665	0.624214	1.109753	1.184476	0.38398	0.399634	0.661876	0.297811	0.33613
3 min	0.700568	0.818906	0.824877	1.235106	1.312686	0.546653	0.571913	0.908192	0.418247	0.4597
4 min	0.88758	1.026637	1.008798	1.351356	1.434061	0.698393	0.73199	1.130932	0.528877	0.57721
5 min	1.06179	1.212562	1.181936	1.460631	1.548873	0.837494	0.881334	1.33405	0.62801	0.68869
204.5 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.411578	0.534649	0.409561	1.042248	0.977062	1.132618	0.870266	0.34278	0.4178	0.45982
2 min	0.624214	0.828774	0.657076	1.72172	1.109753	1.289452	0.888636	0.593725	0.624568	0.75602
3 min	0.824877	1.095027	0.841081	2.405085	1.235106	1.436688	0.906303	0.928906	0.83987	0.90587
4 min	1.008798	1.338607	1.007319	3.2345	1.351356	1.573813	0.92442	1.16537	1.0088	1.05743
5 min	1.181936	1.556883	1.162938	3.65467	1.460631	1.703852	0.942526	1.214362	1.18128	1.25733
255.6 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.163465	0.304731	0.399482	0.072382	0.507572	0.649887	0.539649	0.478956	0.327894	0.2439
2 min	0.301388	0.44971	0.579965	0.182122	0.79617	0.986566	0.923537	0.67845	0.468921	0.45689
3 min	0.431836	0.576334	0.7453	0.287766	1.057329	1.291838	1.272385	0.894821	0.68392	0.78943
4 min	0.553647	0.700386	0.897622	0.387947	1.291838	1.567477	1.589471	1.26733	0.789032	0.90458
5 min	0.673951	0.845805	1.043112	0.484356	1.512174	1.836475	1.871736	1.56201	0.81239	1.0583
511.3 µg L^{-1}										
time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.219327	0.266947	0.327678	0.350998	0.02081	0.31938	0.459346	0.668968	0.432442	0.12378
2 min	0.433335	0.473167	0.458036	0.515273	0.028369	0.5383	0.643325	0.96649	0.618388	0.14482
3 min	0.631108	0.664827	0.581985	0.670777	0.037408	0.743934	0.807949	1.248589	0.79022	0.1651
4 min	0.814218	0.844545	0.700259	0.815064	0.045741	0.937396	0.965685	1.547566	0.955609	0.18655
5 min	0.986821	1.014197	0.814241	0.953058	0.056071	1.122665	1.11235	1.783996	1.129218	0.20771

Calculated values normalised on protein content

24 h	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**
control	0.07	0.08	833	0.01	0.06	157	0.01	0.06	156
102.2 µg L^{-1}	0.04	0.03	1257	0.03	0.11	221	0.01	0.08	222
153.3 µg L^{-1}	0.06	0.09	689	0.05	0.11	432	0.04	0.08	501
204.5 µg L^{-1}	0.06	0.12	489	0.05	0.15	304	0.05	0.09	587
255.6 µg L^{-1}	0.02	0.03	943	0.02	0.12	132	0.05	0.09	542
511.3 µg L^{-1}	0.04	0.03	1295	0.13	0.11	1205	0.09	0.09	1023
control	0.09	0.08	1158	0.02	0.07	261	0.03	0.08	324
102.2 µg L^{-1}	0.03	0.02	1431	0.02	0.14	164	0.02	0.08	346
153.3 µg L^{-1}	0.07	0.06	1231	0.02	0.09	218	0.07	0.13	543
204.5 µg L^{-1}	0.03	0.07	403	0.03	0.08	365	0.04	0.11	374
255.6 µg L^{-1}	/	/	/	0.01	0.08	184	0.01	0.11	133
511.3 µg L^{-1}	0.04	0.03	1038	0.08	0.08	1034	0.08	0.11	689
control	0.03	0.07	380	0.03	0.10	313			
102.2 µg L^{-1}	0.03	0.13	230	0.03	0.12	217			
153.3 µg L^{-1}	0.03	0.12	248	0.04	0.08	456			
204.5 µg L^{-1}	0.05	0.10	509	0.02	0.06	309			
255.6 µg L^{-1}	0.03	0.13	192	0.03	0.16	180			
511.3 µg L^{-1}	0.02	0.10	242	0.06	0.12	459			
control	0.03	0.08	351	0.03	0.11	258			
102.2 µg L^{-1}	0.02	0.05	400	0.02	0.08	306			
153.3 µg L^{-1}	0.05	0.12	456	0.02	0.07	340			
204.5 µg L^{-1}	0.05	0.10	476	0.01	0.03	202			
255.6 µg L^{-1}	0.03	0.14	139	0.01	0.05	136			
511.3 µg L^{-1}	0.03	0.08	349	0.08	0.08	980			

Table 31: GST activity in* G. fossarum *exposed to IMI (A), Confidor 200SL (B) and 6CNA (C) for 24 h. Representative absorbance (AU) values of whole 5 min measurement (selected for every min) and data normalised on total protein content (nmol/min/mg protein). (n=10), (continued).

GST

24 h - C

Control

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.146236	0.042268	0.118228	-0.025388	0.238809	0.225794	0.268316	0.37774	0.256141	0.13408
2 min	0.215348	0.092	0.180029	0.015482	0.32088	0.265945	0.313015	0.462245	0.24998	0.21085
3 min	0.277997	0.13941	0.234261	0.054798	0.348072	0.387399	0.42122	0.518991	0.249274	0.33364
4 min	0.333979	0.179978	0.279339	0.102651	0.3573	0.409343	0.44673	0.563051	0.362658	0.38016
5 min	0.384914	0.21773	0.322515	0.129369	0.457125	0.444866	0.424734	0.593668	0.377037	0.40418

102.2 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.12955	0.128328	0.149784	0.156438	0.340086	0.646463	0.736234	0.447378	1.094399	0.48672
2 min	0.196323	0.200735	0.216454	0.227732	0.441932	0.856097	1.082817	0.805987	1.072066	0.67022
3 min	0.252649	0.260363	0.281522	0.28501	0.621114	1.02245	1.620269	1.267982	1.426711	0.83406
4 min	0.29972	0.299787	0.332891	0.340268	0.837298	1.066789	2.06054	1.509875	1.714141	1.06496
5 min	0.339826	0.363025	0.37819	0.384669	1.694785	1.086095	2.741782	2.569846	2.112663	1.15584

153.3 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.4604	0.4584	0.4529	0.8663	0.481	0.792	0.198045	0.1909	0.2426	0.20689
2 min	0.6432	0.552	0.6775	1.0951	0.667	0.9934	0.349859	0.2552	0.3258	0.33563
3 min	0.8153	0.6526	0.9255	1.3347	0.8494	1.1812	0.434303	0.3208	0.4047	0.45678
4 min	0.9551	0.7609	1.0706	1.5821	1.0103	1.347	0.548586	0.389	0.4888	0.57123
5 min	1.0785	0.9093	1.1767	1.818	1.1224	1.4802	0.693385	0.4573	0.5768	0.6896

204.5 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.8046	0.4861	0.237564	0.293934	0.1423	0.0924	0.0802	0.5305	0.4554	0.4582
2 min	1.0142	0.7114	0.417888	0.487141	0.1499	0.0936	0.085	0.9098	0.8349	0.7232
3 min	1.2352	0.9174	0.523421	0.623672	0.1538	0.0958	0.0891	1.0502	1.0258	0.9712
4 min	1.452	1.1393	0.64742	0.790631	0.1623	0.0969	0.0937	1.2966	1.1768	1.05892
5 min	1.6715	1.3467	0.800012	0.975637	0.17	0.0976	0.0981	1.6087	1.5041	1.3239

255.6 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	1.362637	0.143184	0.133422	0.167281	0.705267	0.517212	0.584282	0.615233	0.533033	0.69791
2 min	1.431397	0.193688	0.213913	0.224842	0.971772	0.721539	0.861622	0.854004	0.744021	1.0215
3 min	1.500165	0.23797	0.282359	0.272417	1.297737	0.929441	1.133561	1.146285	0.93774	1.38988
4 min	1.589687	0.27838	0.343447	0.31585	1.509149	1.151035	1.367819	1.45848	1.161672	2.08173
5 min	1.619464	0.313766	0.408222	0.354798	1.76466	1.296845	1.800128	1.679468	1.375759	2.25647

511.3 µg L^{-1}

time	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*	*AU*
1 min	0.252108	0.162706	0.235301	0.185771	0.45971	0.487406	0.746899	0.413927	0.454748	0.27377
2 min	0.313766	0.204598	0.330724	0.253613	0.665818	0.708818	1.020324	0.572275	0.680329	0.29711
3 min	0.369359	0.242534	0.414906	0.310934	0.847307	0.91868	1.87416	0.722131	0.829636	0.37389
4 min	0.415519	0.275198	0.486956	0.361553	1.014473	1.147863	2.360805	0.863753	1.214336	0.44814
5 min	0.459547	0.307573	0.552477	0.419567	1.170572	1.384937	3.28585	1.446372	1.623092	0.45919

Calculated values normalised on protein content

24 h	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**	**µmol/min**	**mg protein sample**	**nmol/min/mg prot**
control	0.07	0.08	833	0.01	0.06	157	0.01	0.06	156
102.2 µg L^{-1}	0.01	0.09	112	0.05	0.07	763	0.04	0.08	599
153.3 µg L^{-1}	0.04	0.08	523	0.03	0.07	382	0.01	0.12	105
204.5 µg L^{-1}	0.05	0.07	654	0.03	0.07	398	0.01	0.03	346
255.6 µg L^{-1}	0.01	0.09	155	0.04	0.06	700	0.03	0.06	588
511.3 µg L^{-1}	0.01	0.06	156	0.03	0.07	454	0.05	0.06	822
control	0.09	0.08	1158	0.02	0.07	261	0.03	0.08	324
102.2 µg L^{-1}	0.01	0.08	144	0.01	0.08	153	0.03	0.05	561
153.3 µg L^{-1}	0.05	0.07	654	0.02	0.09	255	0.03	0.04	607
204.5 µg L^{-1}	0.04	0.06	702	0.07	0.09	765	0.04	0.08	456
255.6 µg L^{-1}	0.01	0.08	105	0.03	0.06	514	0.08	0.08	970
511.3 µg L^{-1}	0.01	0.07	95	0.04	0.06	626	0.02	0.07	322
control	0.03	0.07	380	0.03	0.10	313			
102.2 µg L^{-1}	0.02	0.13	190	0.09	0.12	776			
153.3 µg L^{-1}	0.06	0.11	571	0.05	0.10	502			
204.5 µg L^{-1}	0.05	0.09	504	0.07	0.12	601			
255.6 µg L^{-1}	0.03	0.13	208	0.05	0.06	805			
511.3 µg L^{-1}	0.02	0.14	113	0.12	0.08	1638			
control	0.03	0.08	351	0.03	0.11	258			
102.2 µg L^{-1}	0.01	0.08	142	0.09	0.12	802			
153.3 µg L^{-1}	0.03	0.09	312	0.02	0.09	201			
204.5 µg L^{-1}	0.04	0.08	502	0.05	0.08	573			
255.6 µg L^{-1}	0.05	0.10	495	0.05	0.08	594			
511.3 µg L^{-1}	0.09	0.13	687	0.04	0.06	597			

Table 32: Absorbance (AU) values for LP measurements in* G. fossarum *exposed to IMI (A), Confidor 200SL (B) and 6CNA (C) for 24 h and data normalised on total protein content (AU final), n=10.

LP

24 h - A

	AU 535 nm	*AU* 600 nm	*AU* 280 nm	***AU final***		*AU* 535 nm	*AU* 600 nm	*AU* 280 nm	***AU final***
control	0.065	0.025	0.28	**0.14**	**control**	0.0664	0.04	0.24	**0.11**
control	0.039	0.012	0.287	**0.09**	**control**	0.0792	0.06	0.192	**0.1**
control	0.07164	0.009	0.261	**0.24**	**control**	0.15072	0.12	0.192	**0.16**
control	0.11328	0.08	0.208	**0.16**	**control**	0.1266	0.09	0.183	**0.2**
control	0.04808	0.032	0.201	**0.08**	**control**	0.11768	0.09	0.173	**0.16**
102.2 µg L^{-1}	0.03792	0.014	0.184	**0.13**	**102.2 µg L^{-1}**	0.0164	0.006	0.08	**0.13**
102.2 µg L^{-1}	0.0438	0.015	0.18	**0.16**	**102.2 µg L^{-1}**	0.09496	0.07	0.096	**0.26**
102.2 µg L^{-1}	0.18835	0.02	0.185	**0.91**	**102.2 µg L^{-1}**	0.1332	0.07	0.08	**0.79**
102.2 µg L^{-1}	0.23571	0.016	0.173	**1.27**	**102.2 µg L^{-1}**	0.06452	0.05	0.121	**0.12**
102.2 µg L^{-1}	0.07171	0.05	0.167	**0.13**	**102.2 µg L^{-1}**	0.13058	0.05	0.102	**0.79**
153.3 µg L^{-1}	0.04838	0.011	0.267	**0.14**	**153.3 µg L^{-1}**	0.08936	0.01	0.248	**0.32**
153.3 µg L^{-1}	0.02204	0.008	0.156	**0.09**	**153.3 µg L^{-1}**	0.0394	0.001	0.384	**0.1**
153.3 µg L^{-1}	0.05309	0.008	0.167	**0.27**	**153.3 µg L^{-1}**	0.02072	0.002	0.234	**0.08**
153.3 µg L^{-1}	0.07132	0.007	0.201	**0.32**	**153.3 µg L^{-1}**	0.04972	0.001	0.203	**0.24**
153.3 µg L^{-1}	0.09617	0.05	0.243	**0.19**	**153.3 µg L^{-1}**	0.0403	0.007	0.222	**0.15**
204.5 µg L^{-1}	0.06175	0.014	0.191	**0.25**	**204.5 µg L^{-1}**	0.06192	0.012	0.312	**0.16**
204.5 µg L^{-1}	0.09889	0.003	0.223	**0.43**	**204.5 µg L^{-1}**	0.0628	0.004	0.28	**0.21**
204.5 µg L^{-1}	0.03163	0.013	0.207	**0.09**	**204.5 µg L^{-1}**	0.00968	0.005	0.234	**0.02**
204.5 µg L^{-1}	0.03338	0.004	0.226	**0.13**	**204.5 µg L^{-1}**	0.03036	0.006	0.203	**0.12**
204.5 µg L^{-1}	0.03616	0.004	0.201	**0.16**	**204.5 µg L^{-1}**	0.12314	0.1	0.178	**0.13**
255.6 µg L^{-1}	0.046	0.017	0.195	**0.15**	**255.6 µg L^{-1}**	0.06028	0.003	0.179	**0.32**
255.6 µg L^{-1}	0.035	0.011	0.195	**0.12**	**255.6 µg L^{-1}**	0.0404	0.002	0.192	**0.2**
255.6 µg L^{-1}	0.15514	0.07	0.198	**0.43**	**255.6 µg L^{-1}**	0.09654	0.06	0.203	**0.18**
255.6 µg L^{-1}	0.12984	0.06	0.194	**0.36**	**255.6 µg L^{-1}**	0.0708	0.03	0.204	**0.2**
255.6 µg L^{-1}	0.08328	0.06	0.194	**0.12**	**255.6 µg L^{-1}**	0.03764	0.002	0.297	**0.12**
511.3 µg L^{-1}	0.05136	0.021	0.276	**0.11**	**511.3 µg L^{-1}**	0.0516	0.006	0.24	**0.19**
511.3 µg L^{-1}	0.081	0.015	0.2	**0.33**	**511.3 µg L^{-1}**	0.04532	0.011	0.264	**0.13**
511.3 µg L^{-1}	0.0312	0.004	0.16	**0.17**	**511.3 µg L^{-1}**	0.03342	0.005	0.203	**0.14**
511.3 µg L^{-1}	0.05136	0.021	0.276	**0.11**	**511.3 µg L^{-1}**	0.03842	0.008	0.234	**0.13**
511.3 µg L^{-1}	0.05053	0.002	0.211	**0.23**	**511.3 µg L^{-1}**	0.05195	0.02	0.213	**0.15**

24 h - B

	AU 535 nm	*AU* 600 nm	*AU* 280 nm	***AU final***		*AU* 535 nm	*AU* 600 nm	*AU* 280 nm	***AU final***
102.2 µg L^{-1}	0.021	0.006	0.098	**0.15**	**102.2 µg L^{-1}**	0.01	0.002	0.112	**0.07**
102.2 µg L^{-1}	0.0164	0.002	0.18	**0.08**	**102.2 µg L^{-1}**	0.0796	0.07	0.096	**0.1**
102.2 µg L^{-1}	0.01318	0.001	0.174	**0.07**	**102.2 µg L^{-1}**	0.017	0.002	0.209	**0.07**
102.2 µg L^{-1}	0.01288	0.001	0.132	**0.09**	**102.2 µg L^{-1}**	0.05968	0.05	0.121	**0.08**
102.2 µg L^{-1}	0.06503	0.05	0.167	**0.09**	**102.2 µg L^{-1}**	0.008	0.004	0.2	**0.02**
153.3 µg L^{-1}	0.02435	0.011	0.267	**0.05**	**153.3 µg L^{-1}**	0.008	0.01	0.248	**0.08**
153.3 µg L^{-1}	0.0158	0.008	0.156	**0.05**	**153.3 µg L^{-1}**	0.008	0.001	0.384	**0.14**
153.3 µg L^{-1}	0.0247	0.008	0.167	**0.1**	**153.3 µg L^{-1}**	0.008	0.002	0.234	**0.133**
153.3 µg L^{-1}	0.01102	0.007	0.201	**0.02**	**153.3 µg L^{-1}**	0.008	0.001	0.203	**0.012**
153.3 µg L^{-1}	0.05729	0.05	0.243	**0.03**	**153.3 µg L^{-1}**	0.008	0.007	0.222	**0.12**
204.5 µg L^{-1}	0.01782	0.014	0.191	**0.02**	**204.5 µg L^{-1}**	0.008	0.012	0.312	**0.08**
204.5 µg L^{-1}	0.01192	0.003	0.223	**0.04**	**204.5 µg L^{-1}**	0.008	0.004	0.28	**0.08**
204.5 µg L^{-1}	0.03163	0.013	0.207	**0.09**	**204.5 µg L^{-1}**	0.008	0.005	0.234	**0.07**
204.5 µg L^{-1}	0.00852	0.004	0.226	**0.02**	**204.5 µg L^{-1}**	0.008	0.006	0.203	**0.02**
204.5 µg L^{-1}	0.02812	0.004	0.201	**0.12**	**204.5 µg L^{-1}**	0.008	0.1	0.178	**0.09**
255.6 µg L^{-1}	0.013	0.002	0.205	**0.5**	**255.6 µg L^{-1}**	0.013	0.001	0.149	**0.08**
255.6 µg L^{-1}	0.0227	0.011	0.195	**0.06**	**255.6 µg L^{-1}**	0.011	0.002	0.124	**0.08**
255.6 µg L^{-1}	0.047	0.011	0.0472	**0.76**	**255.6 µg L^{-1}**	0.009	0.001	0.11	**0.07**
255.6 µg L^{-1}	0.01	0.004	0.107	**0.05**	**255.6 µg L^{-1}**	0.009	0.001	0.11	**0.72**
255.6 µg L^{-1}	0.0988	0.06	0.194	**0.2**	**255.6 µg L^{-1}**	0.30494	0.002	0.297	**1.02**
511.3 µg L^{-1}	0.021	0.004	0.149	**0.11**	**511.3 µg L^{-1}**	0.015	0.002	0.151	**0.09**
511.3 µg L^{-1}	0.025	0.004	0.115	**0.18**	**511.3 µg L^{-1}**	0.02624	0.011	0.254	**0.06**
511.3 µg L^{-1}	0.0216	0.004	0.16	**0.11**	**511.3 µg L^{-1}**	0.0165	0.005	0.23	**0.05**
511.3 µg L^{-1}	0.015	0.002	0.194	**0.07**	**511.3 µg L^{-1}**	0.03572	0.008	0.231	**0.12**
511.3 µg L^{-1}	0.01	0.001	0.147	**0.06**	**511.3 µg L^{-1}**	0.04343	0.02	0.213	**0.11**

Table 32: Absorbance (AU) values for LP measurements in* G. fossarum *exposed to IMI (A), Confidor 200SL (B) and 6CNA (C) for 24 h and data normalised on total protein content (AU final), n=10, (continued).

LP

24 h - C

	AU 535 nm	*AU* 600 nm	*AU* 280 nm	***AU final***		*AU* 535 nm	*AU* 600 nm	*AU* 280 nm	***AU final***
control	0.065	0.025	0.28	**0.14**	**control**	0.0664	0.04	0.24	**0.11**
control	0.039	0.012	0.287	**0.09**	**control**	0.0792	0.06	0.192	**0.1**
control	0.07164	0.009	0.261	**0.24**	**control**	0.15072	0.12	0.192	**0.16**
control	0.11328	0.08	0.208	**0.16**	**control**	0.1266	0.09	0.183	**0.2**
control	0.04808	0.032	0.201	**0.08**	**control**	0.11768	0.09	0.173	**0.16**
102.2 µg L^{-1}	0.021	0.006	0.2382	**0.06**	**102.2 µg L^{-1}**	0.02	0.002	0.186	**0.01**
102.2 µg L^{-1}	0.05001	0.001	0.2883	**0.17**	**102.2 µg L^{-1}**	0.0384	0	0.192	**0.2**
102.2 µg L^{-1}	0.0717	0.008	0.245	**0.26**	**102.2 µg L^{-1}**	0.014	0.001	0.13	**0.1**
102.2 µg L^{-1}	0.0197	0	0.197	**0.1**	**102.2 µg L^{-1}**	0.004	0.001	0.161	**0.01**
102.2 µg L^{-1}	0.008	0	0.185	**0.04**	**102.2 µg L^{-1}**	/	/	/	**/**
153.3 µg L^{-1}	0.00418	0.002	0.218	**0.01**	**153.3 µg L^{-1}**	0.0053	0.002	0.165	**0.02**
153.3 µg L^{-1}	0.03372	0.002	0.244	**0.13**	**153.3 µg L^{-1}**	0.0518	0.01	0.209	**0.2**
153.3 µg L^{-1}	0.001	0.001	0.212	**0**	**153.3 µg L^{-1}**	0.02082	0.003	0.198	**0.09**
153.3 µg L^{-1}	0.04232	0.002	0.288	**0.14**	**153.3 µg L^{-1}**	0.05	0.05	0.184	**0**
153.3 µg L^{-1}	0.03743	0	0.197	**0.19**	**153.3 µg L^{-1}**	0.03812	0.03	0.203	**0.04**
204.5 µg L^{-1}	0.01611	0	0.179	**0.09**	**204.5 µg L^{-1}**	0.05656	0.05	0.164	**0.04**
204.5 µg L^{-1}	0.00382	0	0.191	**0.02**	**204.5 µg L^{-1}**	0.03107	0.02	0.123	**0.09**
204.5 µg L^{-1}	0.02029	0.003	0.133	**0.13**	**204.5 µg L^{-1}**	0.09248	0.08	0.156	**0.08**
204.5 µg L^{-1}	0.02004	0.001	0.136	**0.14**	**204.5 µg L^{-1}**	0.01972	0.001	0.208	**0.09**
204.5 µg L^{-1}	0.0157	0.002	0.137	**0.1**	**204.5 µg L^{-1}**	0.00498	0.001	0.199	**0.02**
255.6 µg L^{-1}	0.009	0.001	0.208	**0.04**	**255.6 µg L^{-1}**	0.02392	0	0.184	**0.13**
255.6 µg L^{-1}	0.007	0.002	0.265	**0.02**	**255.6 µg L^{-1}**	0.0163	0.002	0.143	**0.1**
255.6 µg L^{-1}	0.004	0.002	0.264	**0.08**	**255.6 µg L^{-1}**	0.005	0	0.181	**0.03**
255.6 µg L^{-1}	0.007	0.002	0.265	**0.01**	**255.6 µg L^{-1}**	0.03277	0.03	0.0231	**0.12**
255.6 µg L^{-1}	0.0434	0.02	0.26	**0.09**	**255.6 µg L^{-1}**	0.002	0	0.05	**0.04**
511.3 µg L^{-1}	0.005	0.002	0.257	**0.01**	**511.3 µg L^{-1}**	0.009	0	0.1799	**0.05**
511.3 µg L^{-1}	0.004	0.001	0.239	**0.01**	**511.3 µg L^{-1}**	0.014	0	0.21	**0.07**
511.3 µg L^{-1}	0.004	0.001	0.258	**0.01**	**511.3 µg L^{-1}**	0	0	0.217	**0**
511.3 µg L^{-1}	0.00305	0.001	0.205	**0.01**	**511.3 µg L^{-1}**	0.003	0	0.26	**0.01**
511.3 µg L^{-1}	0.006	0	0.206	**0.03**	**511.3 µg L^{-1}**	0	0	0.199	**0**

Table 33: Values of AChE, CAT and GST activity and LP measurements in* G. fossarum *exposed to lower concentrations of IMI/Confidor 200SL (6.3 – 51.1 µg L^{-1}) and 6CNA (3.9 – 31.4 µg L^{-1}) for 24 h. Data are presented as values normalised on total protein content; AChE and CAT (µmol/min/mg protein), GST (nmol/min/mg protein) and LP (AU). (n=10).

G. fossarum

n=10

Mean values — ***IMI/Confidor 200SL***

µg L^{-1}	AChE	CAT	GST	LP
control	71.17	19.06	427.10	0.22
control	70.82	19.52	422.03	0.18
control	70.59	21.05	416.21	0.21
control	70.96	21.81	417.68	0.21
control	71.56	22.07	417.87	0.20
control	71.12	22.59	401.71	0.21
control	70.41	20.92	407.89	0.18
control	71.02	19.14	421.68	0.22
control	71.51	19.06	421.22	0.20
control	71.37	21.25	420.42	0.21
DMSO+NMP	71.67	20.15	431.33	0.22
DMSO+NMP	71.99	19.71	426.94	0.23
DMSO+NMP	70.27	19.70	411.76	0.21
DMSO+NMP	68.74	20.65	420.03	0.22
DMSO+NMP	68.50	19.25	430.27	0.23
DMSO+NMP	70.23	20.78	401.70	0.22
DMSO+NMP	70.75	19.38	400.58	0.21
DMSO+NMP	70.49	21.63	417.07	0.21
6.3	71.90	22.10	403.48	0.18
6.3	71.92	22.33	420.24	0.18
6.3	70.09	21.72	410.12	0.19
6.3	70.06	21.77	403.10	0.19
6.3	71.71	20.17	409.34	0.19
6.3	70.70	19.93	403.79	0.19
6.3	70.43	21.23	406.35	0.20
6.3	70.10	21.88	407.96	0.23
6.3	70.22	20.39	402.38	0.19
6.3	70.79	21.62	413.96	0.18
12.7	70.61	19.22	405.72	0.21
12.7	71.03	20.36	399.98	0.21
12.7	70.15	20.37	425.08	0.18
12.7	71.90	21.78	426.87	0.22
12.7	70.91	22.87	412.12	0.18
12.7	71.21	22.06	425.85	0.20
12.7	71.74	22.45	429.09	0.20
12.7	70.95	21.73	427.49	0.20
12.7	71.00	20.76	423.08	0.18
12.7	71.03	19.11	416.61	0.23
25.5	71.38	22.50	404.97	0.18
25.5	70.13	19.16	410.94	0.18
25.5	71.45	19.44	416.23	0.22
25.5	71.64	21.09	407.85	0.21
25.5	70.58	21.02	399.60	0.22
25.5	71.48	21.39	411.15	0.22
25.5	71.77	19.23	398.98	0.22
25.5	70.67	19.76	430.80	0.19
25.5	70.84	20.78	423.69	0.21
25.5	71.96	19.80	412.25	0.18
51.1	70.62	20.44	427.62	0.21
51.1	70.26	22.42	426.68	0.18
51.1	70.70	20.42	413.06	0.19
51.1	70.29	21.83	414.38	0.22
51.1	70.76	22.08	414.35	0.19
51.1	71.19	20.49	400.77	0.23
51.1	71.15	22.17	418.82	0.22
51.1	71.63	19.29	415.75	0.18
51.1	70.88	21.92	401.69	0.19
51.1	71.09	20.90	401.68	0.21

Raw data — ***6CNA***

µg L^{-1}	AChE	CAT	GST	LP
3.9	71.68	19.98	413.02	0.22
3.9	71.82	20.74	404.56	0.19
3.9	71.98	22.21	403.74	0.20
3.9	70.73	20.36	418.50	0.23
3.9	71.34	22.35	411.47	0.19
3.9	70.73	20.12	430.45	0.21
3.9	70.86	22.47	418.61	0.21
3.9	71.78	21.25	401.11	0.22
3.9	70.75	19.64	429.41	0.23
3.9	70.54	20.96	407.59	0.18
7.8	71.58	22.01	399.10	0.22
7.8	71.14	21.83	420.90	0.21
7.8	70.00	21.68	405.41	0.23
7.8	71.34	21.44	410.68	0.19
7.8	71.72	19.69	402.85	0.21
7.8	71.31	22.07	416.86	0.20
7.8	70.36	21.14	430.15	0.20
7.8	70.47	21.78	429.14	0.21
7.8	70.36	19.33	414.66	0.22
7.8	71.14	21.14	419.08	0.21
15.7	70.92	19.60	416.28	0.23
15.7	70.28	21.30	428.16	0.23
15.7	71.17	21.48	407.42	0.21
15.7	71.32	20.26	416.09	0.23
15.7	71.24	22.05	419.91	0.22
15.7	70.78	21.23	411.91	0.22
15.7	71.59	21.45	416.39	0.20
15.7	70.80	22.21	409.72	0.22
15.7	71.64	19.86	401.17	0.21
15.7	71.52	20.76	422.52	0.19
31.4	71.10	21.65	398.82	0.19
31.4	70.64	21.99	422.53	0.23
31.4	70.90	21.36	418.10	0.22
31.4	70.79	22.83	413.89	0.20
31.4	71.05	21.15	411.08	0.18
31.4	71.40	20.77	406.40	0.22
31.4	70.94	21.48	406.65	0.23
31.4	71.19	22.91	410.36	0.19
31.4	70.01	19.89	399.44	0.23
31.4	71.50	21.58	410.95	0.22

Table 34: Fluorescence (FU) values for MXR activity measurements in* G. fossarum *exposed to IMI/Confidor 200SL (0.7 and 7.6 mg L^{-1}) or 6CNA (0.4 and 4.7 mg L^{-1}) and copper (0.75, 3 and 10 µg L^{-1}) for 24 h and data normalised on total animal's weight (n=3).

MXR

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Chemical (mg)	*FU*	*FU*	*FU*	*Mean FU*	*Weight (mg)*	*FU/mg*
control	5859	5938	5985	5927.33	26	227.974
control	9231	9077	8757	9021.67	23	392.246
control	6299	6762	6620	6560.33	26	252.321
control	4607	4846	4826	4759.67	35	135.99
control	5569	5392	5694	5551.67	27	205.617
CYC	18099	17825	18057	17993.67	35	514.105
CYC	14241	15206	14453	14633.33	23	636.232
CYC	9457	10253	10524	10078.00	18	559.889
CYC	6714	6960	6846	6840.00	16	427.5
CYC	11235	12231	11266	11577.33	21	551.302
VER	7025	6429	7056	6836.67	14	488.333
VER	6916	6946	6955	6939.00	26	266.885
VER	11431	11193	12274	11632.67	20	581.633
VER	6384	6267	6304	6318.33	5	1263.67
VER	7254	7159	7043	7152.00	13	550.154
IMI 0.7	4846	4775	5012	4877.67	25	195.107
IMI 0.7	4256	3896	4091	4081.00	25	163.24
IMI 0.7	5748	6579	7083	6470.00	17	380.588
IMI 0.7	8567	8041	7973	8193.67	21	390.175
IMI 0.7	6360	5969	5975	6101.33	22	277.333
IMI 7.6	4510	4148	4496	4384.67	21	208.794
IMI 7.6	8431	8709	8115	8418.33	34	247.598
IMI 7.6	8436	8423	8201	8353.33	36	232.037
IMI 7.6	7531	7461	7574	7522.00	24	313.417
IMI 7.6	5174	4638	5072	4961.33	23	215.71
CONFIDOR 0.7	4846	4775	5012	4877.67	31	157.344
CONFIDOR 0.7	4256	3896	4091	4081.00	41	99.5366
CONFIDOR 0.7	5748	6579	7083	6470.00	32	202.188
CONFIDOR 0.7	8567	8041	7973	8193.67	23	356.246
CONFIDOR 0.7	6360	5969	5975	6101.33	32	190.667
CONFIDOR 7.6	7849	8797	8108	8251.33	17	485.373
CONFIDOR 7.6	13280	13531	12829	13213.33	42	314.603
CONFIDOR 7.6	10997	11237	11196	11143.33	38	293.246
CONFIDOR 7.6	9958	10005	10282	10081.67	41	245.894
CONFIDOR 7.6	4333	7676	7415	6474.67	34	190.431
6CNA 0.4	18099	17825	18057	17993.67	22	817.894
6CNA 0.4	14241	15206	14453	14633.33	36	406.481
6CNA 0.4	9457	10253	10524	10078.00	23	438.174
6CNA 0.4	6714	6960	6846	6840.00	28	244.286
6CNA 0.4	11235	12231	11266	11577.33	14	826.952
6CNA 4.7	7025	6429	7056	6836.67	35	195.333
6CNA 4.7	6916	6946	6955	6939.00	26	266.885
6CNA 4.7	11431	11193	12274	11632.67	36	323.13
6CNA 4.7	6384	6267	6304	6318.33	28	225.655
6CNA 4.7	7254	7159	7043	7152.00	21	340.571
Cu 3 µg L^{-1}	7398	7606	7601	7535.00	38	198.289
Cu 3 µg L^{-1}	10354	9455	9452	9753.67	35	278.676
Cu 3 µg L^{-1}	9202	9305	9769	9425.33	33	285.616
Cu 3 µg L^{-1}	8443	7696	12535	9558.00	41	233.122
Cu 3 µg L^{-1}	6007	5404	7343	6251.33	20	312.567
Cu 10 µg L^{-1}	4510	4148	4496	4384.67	41	106.943
Cu 10 µg L^{-1}	8431	8709	8115	8418.33	23	366.014
Cu 10 µg L^{-1}	8436	8423	8201	8353.33	35	238.667
Cu 10 µg L^{-1}	7531	7461	7574	7522.00	32	235.063
Cu 10 µg L^{-1}	5174	4638	5072	4961.33	23	215.71
IMI 0.7 + Cu 3 µg L^{-1}	7503	6372	7149	7008.00	32	219
IMI 0.7 + Cu 3 µg L^{-1}	11172	10955	11097	11074.67	23	481.507
IMI 0.7 + Cu 3 µg L^{-1}	2824	10313	8460	7199.00	36	199.972
IMI 0.7 + Cu 3 µg L^{-1}	9635	10147	10409	10063.67	25	402.547
IMI 0.7 + Cu 3 µg L^{-1}	7263	7466	7707	7478.67	23	325.159
IMI 0.7 + Cu 10 µg L^{-1}	8773	8864	9420	9019.00	28	322.107
IMI 0.7 + Cu 10 µg L^{-1}	11518	11849	17280	13549.00	20	677.45
IMI 0.7 + Cu 10 µg L^{-1}	13028	12474	20861	15454.33	32	482.948
IMI 0.7 + Cu 10 µg L^{-1}	9671	10437	10533	10213.67	27	378.284
IMI 0.7 + Cu 10 µg L^{-1}	7975	8231	8486	8230.67	25	329.227

Table 34: Fluorescence (FU) values for MXR activity measurements in* G. fossarum *exposed to IMI/Confidor 200SL (0.7 and 7.6 mg L^{-1}) or 6CNA (0.4 and 4.6 mg L^{-1}) and copper (0.75, 3 and 10 µg L^{-1}) for 24 h and data normalised on total animal's weight (n=3), (continued).

MXR

II

Chemical (mg)	*FU*	*FU*	*FU*	*Mean FU*	*Weight (mg)*	*FU/mg*
control	7542	7597	7530	7556.33	35	215.895
control	7811	7439	7413	7554.33	29	260.494
control	10523	10553	12116	11064.00	35	316.114
control	10934	10958	11261	11051.00	26	425.038
control	5955	5825	6577	6119.00	27	226.63
CYC	16693	15812	12700	15068.33	26	579.551
CYC	13912	12901	12726	13179.67	27	488.136
CYC	14576	14988	14940	14834.67	34	436.314
CYC	7085	6563	7076	6908.00	33	209.333
CYC	12863	12024	11792	12226.33	18	679.241
VER	7076	7260	7659	7331.67	14	523.69
VER	6788	6610	7068	6822.00	26	262.385
VER	11669	12000	12035	11901.33	20	595.067
VER	9675	9610	9843	9709.33	14	693.524
VER	14049	14954	14574	14525.67	13	1117.36
IMI 0.7	4505	4410	4318	4411.00	15	294.067
IMI 0.7	5121	4986	5256	5121.00	27	189.667
IMI 0.7	8424	8863	8479	8588.67	34	252.608
IMI 0.7	6070	5386	6695	6050.33	18	336.13
IMI 0.7	4679	4574	4704	4652.33	20	232.617
IMI 7.6	7088	6047	4479	5871.33	33	177.919
IMI 7.6	6823	7579	5702	6701.33	35	191.467
IMI 7.6	8402	8049	6417	7622.67	25	304.907
IMI 7.6	5574	5329	4885	5262.67	20	263.133
IMI 7.6	5391	5478	5299	5389.33	20	269.467
CONFIDOR 0.7	8112	8068	7985	8055.00	23	350.217
CONFIDOR 0.7	5646	5464	5663	5591.00	17	328.882
CONFIDOR 0.7	6759	9084	9971	8604.67	24	358.528
CONFIDOR 0.7	9726	9879	9838	9814.33	32	306.698
CONFIDOR 0.7	5302	5172	5134	5202.67	37	140.613
CONFIDOR 7.6	10063	10201	10871	10378.33	30	345.944
CONFIDOR 7.6	5512	5184	5261	5319.00	17	312.882
CONFIDOR 7.6	5387	5088	5133	5202.67	20	260.133
CONFIDOR 7.6	6691	6351	6313	6451.67	35	184.333
CONFIDOR 7.6	4140	4349	4737	4408.67	14	314.905
6CNA 0.4	4810	4558	4975	4781.00	17	281.235
6CNA 0.4	6150	6382	6300	6277.33	18	348.741
6CNA 0.4	8319	7656	8049	8008.00	31	258.323
6CNA 0.4	6921	6951	7221	7031.00	17	413.588
6CNA 4.7	6104	5324	4649	5359.00	32	167.469
6CNA 4.7	7530	7072	8045	7549.00	34	222.029
6CNA 4.7	5004	4707	3894	4535.00	12	377.917
6CNA 4.7	4664	5489	6359	5504.00	19	289.684
Cu 0.75 µg L^{-1}	7398	7606	7601	7535.00	25	301.4
Cu 0.75 µg L^{-1}	10354	9455	9452	9753.67	36	270.935
Cu 0.75 µg L^{-1}	9202	9305	9769	9425.33	26	362.513
Cu 0.75 µg L^{-1}	8443	7696	12535	9558.00	26	367.615
Cu 0.75 µg L^{-1}	6007	5404	7343	6251.33	25	250.053

Table 34: Fluorescence (FU) values for MXR activity measurments in* G. fossarum *exposed to IMI/Confidor 200SL (0.7 and 7.6 mg L^{-1}) or 6CNA (0.4 and 4.6 mg L^{-1}) and copper (0.75, 3 and 10 µg L^{-1}) for 24 h and data normalised on total animal's weight (n=3), (continued).

MXR

II

Chemical (mg)	*FU*	*FU*	*FU*	*Mean FU*	*Weight (mg)*	*FU/mg*
IMI 0.7 + Cu 0.75 µg L^{-1}	10051	8614	10440	9701.667	15	646.778
IMI 0.7 + Cu 0.75 µg L^{-1}	7857	8271	8206	8111.333	22	368.697
IMI 0.7 + Cu 0.75 µg L^{-1}	6847	6757	6331	6645	20	332.25
IMI 0.7 + Cu 0.75 µg L^{-1}	8406	7333	8252	7997	25	319.88
IMI 0.7 + Cu 0.75 µg L^{-1}	3930	3979	3979	3962.667	12	330.222
IMI 0.7 + Cu 3 µg L^{-1}	21065	19993	21218	20758.67	20	1037.93
IMI 0.7 + Cu 3 µg L^{-1}	9321	9275	9565	9387	16	586.688
IMI 0.7 + Cu 3 µg L^{-1}	8579	8316	8632	8509	20	425.45
IMI 0.7 + Cu 3 µg L^{-1}	10373	10078	10170	10207	41	248.951
IMI 0.7 + Cu 3 µg L^{-1}	8670	8919	8359	8649.333	24	360.389
IMI 0.7 + Cu 10 µg L^{-1}	9885	9791	11097	10257.67	37	277.234
IMI 0.7 + Cu 10 µg L^{-1}	10076	9314	10202	9864	29	340.138
IMI 0.7 + Cu 10 µg L^{-1}	8807	8501	9295	8867.667	30	295.589
IMI 0.7 + Cu 10 µg L^{-1}	7670	7291	6490	7150.333	20	357.517
IMI 0.7 + Cu 10 µg L^{-1}	6528	6289	6511	6442.667	19	339.088
6CNA 0.4 + Cu 0.75 µg L^{-1}	10413	10402	10590	10468.33	23	455.145
6CNA 0.4 + Cu 0.75 µg L^{-1}	11331	11737	12093	11720.33	20	586.017
6CNA 0.4 + Cu 0.75 µg L^{-1}	13490	12953	13089	13177.33	28	470.619
6CNA 0.4 + Cu 0.75 µg L^{-1}	9252	8759	5609	7873.333	20	393.667
6CNA 0.4 + Cu 0.75 µg L^{-1}	8339	8645	7021	8001.667	20	400.083
6CNA 0.4 + Cu 3 µg L^{-1}	8458	8406	9406	8756.667	22	398.03
6CNA 0.4 + Cu 3 µg L^{-1}	8373	8723	8281	8459	18	469.944
6CNA 0.4 + Cu 3 µg L^{-1}	9574	10189	9842	9868.333	27	365.494
6CNA 0.4 + Cu 3 µg L^{-1}	11020	10484	10382	10628.67	28	379.595
6CNA 0.4 + Cu 3 µg L^{-1}	9554	9708	9150	9470.667	20	473.533
6CNA 0.4 + Cu 10 µg L^{-1}	8436	7834	7652	7974	33	241.636
6CNA 0.4 + Cu 10 µg L^{-1}	9205	9023	8953	9060.333	23	393.928
6CNA 0.4 + Cu 10 µg L^{-1}	11432	10897	10871	11066.67	13	851.282
6CNA 0.4 + Cu 10 µg L^{-1}	9223	9143	9369	9245	14	660.357
6CNA 0.4 + Cu 10 µg L^{-1}	6897	6970	7162	7009.667	16	438.104
Cu 3 µg L^{-1}	5008	4555	4856	4806.333	18	267.019
Cu 3 µg L^{-1}	5883	6261	6644	6262.667	22	284.667
Cu 3 µg L^{-1}	6719	6524	6687	6643.333	18	369.074
Cu 3 µg L^{-1}	15341	14956	15695	15330.67	30	511.022
Cu 3 µg L^{-1}	8124	7732	4547	6801	23	295.696
Cu 10 µg L^{-1}	7527	7397	8179	7701	28	275.036
Cu 10 µg L^{-1}	10841	10541	11875	11085.67	36	307.935
Cu 10 µg L^{-1}	5185	5168	5755	5369.333	15	357.956
Cu 10 µg L^{-1}	5156	4608	4653	4805.667	13	369.667
Cu 10 µg L^{-1}	13806	13428	16417	14550.33	14	1039.31

Table 34: Fluorescence (FU) values for MXR activity measurments in* G. fossarum *exposed to IMI/Confidor 200SL (0.7 and 7.6 mg L^{-1}) or 6CNA (0.4 and 4.6 mg L^{-1}) and copper (0.75, 3 and 10 µg L^{-1}) for 24 h and data normalised on total animal's weight (n=3), (continued).

MXR

III

Chemical (mg)	*Mean FU*	*Weight (mg)*	*FU/mg*	*Chemical (mg)*	*Mean FU*	*Weight (mg)*	*FU/mg*
control	4875.0	38	128.2894737	IMI 0.7 + Cu 0.75 µg L^{-1}	6217.0	27	230.259
control	6304.0	25	252.16	IMI 0.7 + Cu 0.75 µg L^{-1}	16866.0	27	624.667
control	5179.0	22	235.4090909	IMI 0.7 + Cu 0.75 µg L^{-1}	9881.0	31	318.742
control	4949.0	28	176.75	IMI 0.7 + Cu 0.75 µg L^{-1}	7666.0	30	255.533
control	5691.0	26	218.8846154	IMI 0.7 + Cu 0.75 µg L^{-1}	8779.0	30	292.633
CYC	5681.0	32	177.53125	IMI 0.7 + Cu 3 µg L^{-1}	7144.0	23	310.609
CYC	4973.0	18	276.2777778	IMI 0.7 + Cu 3 µg L^{-1}	7157.0	24	298.208
CYC	7842.0	28	280.0714286	IMI 0.7 + Cu 3 µg L^{-1}	7260.0	34	213.529
CYC	6995.0	27	259.0740741	IMI 0.7 + Cu 3 µg L^{-1}	9489.0	24	395.375
CYC	11071.0	22	503.2272727	IMI 0.7 + Cu 3 µg L^{-1}	8136.0	23	353.739
VER	5797.0	24	241.5416667	IMI 0.7 + Cu 10 µg L^{-1}	6534.0	23	284.087
VER	5630.0	20	281.5	IMI 0.7 + Cu 10 µg L^{-1}	8166.0	26	314.077
VER	6675.0	19	351.3157895	IMI 0.7 + Cu 10 µg L^{-1}	8764.0	28	313
VER	6396.0	18	355.3333333	IMI 0.7 + Cu 10 µg L^{-1}	28904.0	34	850.118
VER	7013.0	20	350.65	IMI 0.7 + Cu 10 µg L^{-1}	20856.0	20	1042.8
IMI 0.7	6369.0	32	199.03125	6CNA 0.4 + Cu 0.75 µg L^{-1}	9329.0	24	388.708
IMI 0.7	4690.0	22	213.1818182	6CNA 0.4 + Cu 0.75 µg L^{-1}	11024.0	25	440.96
IMI 0.7	5864.0	24	244.3333333	6CNA 0.4 + Cu 0.75 µg L^{-1}	8096.0	15	539.733
IMI 0.7	5245.0	24	218.5416667	6CNA 0.4 + Cu 0.75 µg L^{-1}	7295.0	15	486.333
IMI 0.7	6156.0	23	267.6521739	6CNA 0.4 + Cu 0.75 µg L^{-1}	7888.0	22	358.545
IMI 7.6	10862.0	27	402.2962963	6CNA 0.4 + Cu 3 µg L^{-1}	5441.0	17	320.059
IMI 7.6	7065.0	17	415.5882353	6CNA 0.4 + Cu 3 µg L^{-1}	9423.0	19	495.947
IMI 7.6	4847.0	22	220.3181818	6CNA 0.4 + Cu 3 µg L^{-1}	6262.0	11	569.273
IMI 7.6	7147.0	26	274.8846154	6CNA 0.4 + Cu 3 µg L^{-1}	8585.0	15	572.333
IMI 7.6	7047.0	24	293.625	6CNA 0.4 + Cu 3 µg L^{-1}	8123.0	27	300.852
CONFIDOR 0.7	6446.0	24	268.5833333	6CNA 0.4 + Cu 10 µg L^{-1}	10058.0	34	295.824
CONFIDOR 0.7	8348.0	25	333.92	6CNA 0.4 + Cu 10 µg L^{-1}	15211.0	32	475.344
CONFIDOR 0.7	6560.0	29	226.2068966	6CNA 0.4 + Cu 10 µg L^{-1}	10811.0	30	360.367
CONFIDOR 0.7	6904.0	27	255.7037037	6CNA 0.4 + Cu 10 µg L^{-1}	13534.0	26	520.538
CONFIDOR 0.7	7216.0	25	288.64	6CNA 0.4 + Cu 10 µg L^{-1}	8184.0	16	511.5
CONFIDOR 7.6	7117.0	31	229.5806452	Cu 3 µg L^{-1}	8236.0	27	305.037
CONFIDOR 7.6	5592.0	24	233	Cu 3 µg L^{-1}	10337.0	19	544.053
CONFIDOR 7.6	8788.0	27	325.4814815	Cu 3 µg L^{-1}	8688.0	26	334.154
CONFIDOR 7.6	5457.0	28	194.8928571	Cu 3 µg L^{-1}	5985.0	18	332.5
CONFIDOR 7.6	6925.0	30	230.8333333	Cu 3 µg L^{-1}	8148.0	23	354.261
6CNA 0.4	5324.0	19	280.2105263	Cu 10 µg L^{-1}	12155.0	27	450.185
6CNA 0.4	10703.0	10	1070.3	Cu 10 µg L^{-1}	12386.0	27	458.741
6CNA 0.4	9669.0	20	483.45	Cu 10 µg L^{-1}	9319.0	23	405.174
6CNA 0.4	5027.0	12	418.9166667	Cu 10 µg L^{-1}	9104.0	26	350.154
6CNA 0.4	6201.0	11	563.7272727	Cu 10 µg L^{-1}	6193.0	30	206.433
6CNA 4.7	9332.0	18	518.4444444				
6CNA 4.7	6644.0	25	265.76				
6CNA 4.7	8014.0	21	381.6190476				
6CNA 4.7	7262.0	24	302.5833333				
6CNA 4.7	6903.0	23	300.1304348				
Cu 0.75 µg L^{-1}	12718.0	28	454.2142857				
Cu 0.75 µg L^{-1}	8523.0	31	274.9354839				
Cu 0.75 µg L^{-1}	12161.0	29	419.3448276				
Cu 0.75 µg L^{-1}	7835.0	24	326.4583333				
Cu 0.75 µg L^{-1}	7032.0	28	251.1428571				

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"« Gutta cavat lapidem, non bis sed saepe cadendo,
Sic homo fit sapiens bis non, sed saepe legendo"

Giordano Bruno

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