Impacts of fungi on marine organisms and human health: the 2009 Australian dust storm associated *Aspergillus sydowii* bloom

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Declaration of Originality

I declare that the material presented in this thesis is original, except where due acknowledgement is given, and has not been accepted for the awards of any other degree of diploma

Aiko Hayashi

February, 2019

Authority of Access

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Anthony J. Richardson (5%) contributed the CPR fungal silks.

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Dedication

To my mother and sister,
Masami Hayashi and Maki Hayashi,
for their support and patience

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Abstract

The incidence of massive *Aspergillus sydowii* marine "fungal slicks" along the east coast of Australia between Brisbane and Sydney in the wake of the 2009 dust storm, covering an area 25-times the surface of England, has raised concerns about marine ecosystem as well as human health impacts. Our current knowledge on the impacts of fungi on marine organisms and human health through seafood consumption is very limited. The present study aimed at: i) Elucidation of pathogenicity of *A. sydowii* against the coral endosymbiont *Symbiodinium* as a model to elucidate the cause of sea fan coral aspergillosis; ii) Impacts of emerging mycotoxins on marine organisms using a fish gill cell line model (RT-gillW1); and iii) Assessment of combined toxicity of mycotoxins and phycotoxins on human cell line models (intestinal HT-29 and neuroblastoma SH-SY5Y).

Dust originated (ASBS), terrestrial (FRR5068) and sea fan coral pathogenic (FK1) *A. sydowii* fungal strains all produced the same set of known *A. sydowii* metabolites, including sydowinin A, sydowinin B, sydowinol, sydonic acid, hydroxysydonic acid and sydonol, but minor metabolites differed between strains. Sydowic acid, sydowinol and sydowinin A adversely affected photophysiological performance (F_v/F_m) of the coral reef dinoflagellate endosymbiont *Symbiodinium*. Moreover, different *Symbiodinium* clades exhibited varying sensitivities to these fungal metabolites, mimicking sensitivity to coral bleaching phenomena in sea fan coral aspergillosis. Re-evaluation of the 2009 dust storm silks confirmed the dominance of *A. sydowii* (73.7%), with varying metabolite profiles, but these all produced sydonic acid. Other minor fungal isolates newly found in this study included *Cladosporium, Penicillium* and other *Aspergillus* species, which suggests potential secondary colonisation of the 2009 dust storm rafts.

Shellfish and 2009 dust storm associated mycotoxins (e.g. patulin, alamethicin, gliotoxin and major *A. sydowii* metabolites) exhibited significant cytotoxicity to the fish gill cell (RT-gillW1) cell line with IC_{50} (inhibitory concentration 50%) values of 0.098 – 103.7 µM. Previously reported combined effect of alamethicin and domoic acid using a larval Diptera bioassay, was not observed when using RT-gillW1 cells. The current study also evaluated the cytotoxicity of shellfish and dust storm associated mycotoxins and algal toxin okadaic acid

(responsible for the syndrome of Diarrhetic Shellfish Poisoning), either alone or in combination, when challenged against human intestinal (HT-29) and neuroblastoma (SH-SY5Y) cell lines. Combinations of okadaic acid, sydowinin A, alamethicin, patulin, and gliotoxin exhibited shifts from antagonism to additive/synergistic interactions with increasing cytotoxicity, with okadaic acid–sydowinol displaying an antagonistic relationship against HT-29 cells. Furthermore, only the okadaic acid–sydowinin A combination showed synergism, while okadaic acid combined with sydowinol, alamethicin, patulin, or with gliotoxin demonstrated antagonism against SH-SY5Y. While Diarrhetic Shellfish Poisoning from okadaic acid and analogues in many parts of the world is considered to be a comparatively minor seafood toxin syndrome, our human cell model studies provide the first insights that synergisms with mycotoxin may aggravate human health impacts. These findings highlight the shortcomings of current regulatory approaches, which do not regulate for mycotoxins in shellfish and treat seafood toxins as if they occur as single toxins.

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Chapter 1. Introduction

The significance of fungi in marine environments is increasingly being recognised. They often have been isolated from marine substrata such as mangroves, sediments and marine organisms (Ein-Gil et al. 2009; Kohlmeyer 1969; Morrison-Gardiner 2002). Their roles in marine environments include as decomposer, parasites and pathogens (Hyde et al. 1998). Furthermore, fungi infect a wide range of marine organisms such as coralline algae (Littler & Littler 1998), loggerhead sea turtles (Sarmiento-Ramírez et al. 2010), corals (Smith et al. 1996), marine fish and prawns (Hatai 2012). The recent emergence of fungal disease in marine ecosystems is arguably related to anthropogenic stressors such as eutrophication, land runoff, climate change and dust storms. These stressors either transport toxigenic fungal strains, or promote the growth of toxigenic fungi in marine environments, while suppressing host immunity (Gleason et al. 2017). Fungi also potentially contaminate shellfish and fish, thereby posing health risks to human consumers. However, despite increasing risks of fungal disease and seafood contaminations, our present knowledge on fungi in marine environments is very limited.

1.1 Dust storms and pathogenic fungal species

In marine ecosystems, dust deposition plays important roles in transferring nutrients and pathogens. Dust blows across large areas of the Atlantic, Pacific and Indian Oceans (Husar, Prospero & Stowe 1997), with soil borne nutrients being transported to marine environments which potentially can stimulate the primary production in some oceanic regions (Karl et al. 2002). For example, dust deposition during the severe 2002 dust storm in Australia increased standing stock levels of chlorophyll concentration in coastal waters by

1.5-2 times (Shaw, Gabric & McTainsh 2008). Furthermore, terrestrial species such as *Aspergillus*, *Cladosporium* and *Penicillium* species have been previously isolated from Caribbean and African dust events (Kellogg et al. 2004; Shinn et al. 2000; Weir-Brush et al. 2004). Therefore, dust was also suggested as one of the major contributors to the outbreak in the Caribbean of sea fan aspergillosis disease associated with *A. sydowii* (Garrison et al. 2003; Shinn et al. 2000). Moreover, significant masses of *Aspergillus sydowii* spores and mycelia (~150,000 spores/m^3) in coastal waters were collected by the Continuous Plankton Recorder (CPR) after the 2009 Australian dust storm (Hallegraeff *et al.*, 2014) (Figure 1.1).

Future projections of dust emissions based on model studies are complex and uncertain (Tegen et al. 2004), however anthropogenically-induced changes in climate and vegetation have significant influences on future dust emissions (Prospero & Lamb 2003; Tegen et al. 2004). Expected increases in drought risk under climate change (Cook, Mankin & Anchukaitis 2018), and reduced vegetation would worsen the impact and increase the frequency of dust storms (Prospero & Lamb 2003; Speer 2013). Furthermore, airborne and potentially pathogenic species such as *Aspergillus*, *Penicillium*, *Cladosporium*, *Fusarium* and *Alternaria* exhibit high salinity, temperature and pH tolerance (Gleason et al. 2017). *Penicillium* exhibits high tolerance of salinity (10-22 %), temperature (4-37 ^oC) and pH (1.5-14) (Dhakar, Sharma & Pandey 2014). Elevated salinity increased mitochondria size, rate of respiration and levels of respiratory enzymes of *A. sydowii* (Parekh & Chhatpar 1989). Furthermore, temperature had a significant effects on the growth rate of pathogenic *A. sydowii* with an optimum of 30 ^oC, while reducing host defences response (Alker, Smith & Kim 2001). Therefore, increasing temperature and acidity under current climate change scenarios might promote their survival and growth in marine environments (Gleason et al. 2017). However, these factors also have adverse effects on host immunity of marine animals (Harvell et al. 2009; Mackenzie et al. 2014). Consequently, fungi related disease in marine ecosystems is predicted to increase under climate change scenarios that increase translocation of fungi and enhance fungal survival growth, and decrease host immunity. Furthermore, the 2009 *A. sydowii* fungal bloom raised the alarm for future marine ecosystem and emerging human health impacts.

Despite the fact that increasing marine ecosystem and human health risks, our current knowledge on fungi in marine environment is limited compared to those in terrestrial environments (Amend et al. 2019). There is a growing evidence which fungi are abundant and diverse in marine environments(Amend et al. 2019). Fungi have been isolated from the surface waters (Taylor & Cunliffe 2016), the deep sea (Orsi, Biddle & Edgcomb 2013), sediments (Orsi, Biddle & Edgcomb 2013), drift wood (Rämä et al. 2014), algae (Wainwright et al. 2017), invertebrates (Ein-Gil et al. 2009) and mammals (Higgins 2000). So far, studies of those fungi have been based on t phylogenetic analysis, and their conclusions of role of fungi in the marine environment remains speculative (Amend, Barshis & Oliver 2012; Gutiérrez, Jara & Pantoja 2016; Littman, Willis & Bourne 2011; Yarden et al. 2007) or unknown (Amend 2014; Amend et al. 2019). Fungal enzymes and metabolites have been well known to be involved in interactions with hosts in terrestrial environments (Meena et al. 2015), and possibly in marine environments as well (Raghukumar & Ravindran 2012). However, current studies tend to focus on the pharmaceutical uses of those metabolites from the marine habitat (Deshmukh, Prakash & Ranjan 2018),but their ecological roles have remain unclear.

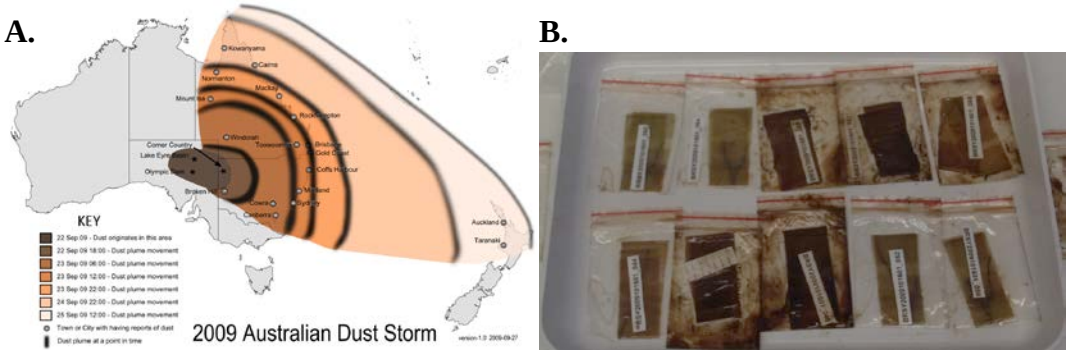

Figure 1.1 Map of the progress of the intensive Australian dust storm in 2009 (from Wikipedia) (A) and photographs of CPR silks after 2009 dust storm (B).

1.2 Sea fan coral aspergillosis

Fungal diseases in marine environments have significant impacts on ecosystem functioning. *A. sydowii* was previously well known as causative agent of sea fan aspergillosis

disease causing declines in colony numbers by 50% loss between 1997 and 2003 (Kim & Harvell 2004) and 20 – 90 % mortalities in the Florida Keys (Bruno *et al.* 2011) (Figure 1.2). Detailed molecular study revealed that pathogenic *A. sydowii* isolates do not form distinct genetic clades with closely related isolates coming from different geographic locations. There was no observed genetic differences between non-pathogenic and pathogenic strains (Rypien, Andras & Harvell 2008). Furthermore, Alker, Smith and Kim (2001) observed no clear differences in temperature effects on growth rates and carbon utilisation patterns between pathogenic and non-pathogenic strains. These genetic and physiological studies supported *Aspergillus* species as opportunistic pathogens, and suggested the absence of a specific pathogenicity and hence indicating that any *A. sydowii* strain could cause aspergillosis. However, only pathogenic strains caused aspergillosis disease while non-pathogenic strains cause no disease symptoms (Geiser et al. 1998). It was observed metabolite profiles differed among pathogenic and non-pathogenic isolates (Malmstrøm et al. 2001). This suggests that fungal secondary metabolites might constitute the difference in *A. sydowii* pathogenicity. However, the roles of secondary metabolites in aspergillosis pathogenicity remain unexplored.

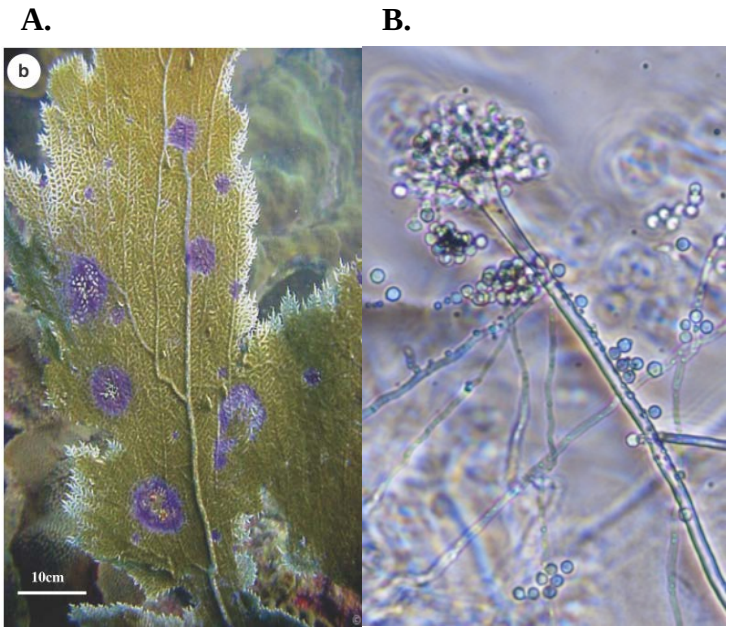

Figure 1.2 Sea fan aspergillosis on the sea fan, *Gorgonia ventalina*. Purple necrotic rings are typical symptoms of aspergillosis (Ein-Gil et al. 2009) (A) and micrograph of causative agent *A. sydowii* (B).

Research into coral disease has mostly focused on etiology or ecological impacts because of their role in coral habitat loss (Correa et al. 2009). Therefore, previous studies on coral disease, including sea fan aspergillosis, lack in depth understanding of interactions that can occur between disease-causing taxa, coral species and the associated dinoflagellate endosymbiont (Correa et al. 2009). Coral dinoflagellate endosymbionts tend to be members of genus *Symbiodinium*, which plays an essential role to corals by providing up to 90% of fixed carbon for metabolism (Muscatine & Porter 1977). Impacts on *Symbiodinium* viability and/or activity therefore reflect coral host susceptibility to disease. For example, yellow band disease involves intracellular *Symbiodinium* infections by *Vibrio* spp. that cause their degradation and subsequent expulsion from coral (Ben-Haim, Zicherman-Keren & Rosenberg 2003). Other coral diseases may also impair photophysiological properties (F_V/F_M) of *Symbiodinium* (Burns, Gregg & Takabayashi 2013). In contrast, the coral disease Acroporid white syndrome does not affect *Symbiodinium*, since infected coral tissues does not exhibit damage to *Symbiodinium* cells as well as no significant effects on symbiont density, mitotic index, or chlorophyll concentrations (Roff et al. 2008). *Symbiodinium* clade diversity is another factor which underpins the host suspectability to disease and disease pathogenicity.

To date, eight *Symbiodinium* genetic clades (A-H) have been characterised (Pochon et al. 2006). These clades exhibit physiological differences (Warner et al. 2006) and could potentially reflect host resistance to temperature stress and colony distribution (Berkelmans & Van Oppen 2006; Iglesias-Prieto et al. 2004). Studies on *Vibrio* bacteria related coral disease and yellow band disease demonstrated that *Symbiodinium* clade A dominated in infected coral tissues while healthy corals harboured either only clade C or both clades A and D (Rouzé et al. 2016; Toller, Rowan & Knowlton 2001). Correa *et al.* (2009) also demonstrated that fewer diseased corals contained *Symbiodinium* clade D, however there was no association between specific *Symbiodinium* clade types and diseased corals. Similarly, Kirk, Ward and Coffroth (2005) reported that *Symbiodinium* clade B were isolated from both healthy and aspergillosis diseased corals hosts. These results suggests there is likely no difference in *Symbiodinium* clades between healthy and infected hosts.

1.3 Mycotoxins as emerging toxins in aquaculture

Fungi in marine environments are also increasingly recognised as aquaculture feed contaminants. Recently aquaculture feeds have been substituted with more economical and sustainable plant materials due to significant growth in aquaculture animal production (Food and Agriculture Organization of the United Nations 2016; Tacon, Hasan & Metian 2011). Typical plant based ingredients for aquaculture feeds include soybean, wheat, maize, corn, rapeseed/canola, cottonseed and rice bran (Gonçalves et al. 2017). These represent favourable growth substrates for numerous toxigenic fungi such as such as *Aspergillus, Fusarium* and *Penicillium* (Jayaraman & Kalyanasundaram 1990; Mills 1990; Murphy et al. 2006). Several studies confirmed that aquaculture feeds are often contaminated with toxigenic fungi and their mycotoxins (Barbosa et al. 2013; Gonçalves et al. 2017; Gonçalves, Naehrer & Santos 2016; Pietsch et al. 2013). Adverse effects of these mycotoxins on fish include reduction of growth and feed efficiency, damage to organs, and neurotoxicity and even mortality (Anater et al. 2016). Impacts on aquatic animals have been investigated less compared to terrestrial livestock animals (Gonçalves, Naehrer & Santos 2016). Furthermore, information on toxicokinetics of mycotoxins in fish is limited to aflatoxin and *Fusarium* mycotoxins. El-Sayed and Khalil (2009) demonstrated that feed borne aflatoxin (~5 µg/kg) accumulates in

the musculature of sea bass (*Dicentrarchus labrax* L.). Furthermore, *Fusarium* mycotoxins, enniatins and beauvericin accumulated in muscle, liver, head and viscera of commercially available sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) (Tolosa et al. 2014). Such carry-over of mycotoxins from fish represents potential health risks for fish consumers, highlighting the need for implementing mycotoxin monitoring and management in aquaculture.

Mycotoxins have also been recognised as new shellfish toxins. The first possible event of mycotoxin causing shellfish toxicity was reported in the early 1990s on the French coast where mouse bioassays detected high levels of shellfish toxicity (Amzil, Marcaillou-Le Baut & Bohec 1996). However, chemical analysis detected no known algal toxins such as PSP (paralytic shellfish poisoning) or DSP (diarrheic shellfish poisoning) toxins, or other chemical pollutants (Amzil, Marcaillou-Le Baut & Bohec 1996). This led to the suggestion of possible involvement of mycotoxins, as toxigenic fungal species had been previously isolated from shellfish in Canada (Brewer, Greenwell & Taylor 1993). Follow up studies confirmed the presence of toxigenic fungal species such as *Aspergillus*, *Penicillium*, *Trichoderma* and *Cladosporium* from shellfish, sediment and seawater from the shellfish farming areas also in France (Sallenave-Namont et al. 2000). Some of those fungal isolates produced toxic metabolites including gliotoxin (Grovel, Pouchus & Verbist 2003), patulin (Vansteelandt et al. 2012), griseofulvin (Petit et al. 2004) and peptaibol (Poirier, Montagu, et al. 2007). Gliotoxin accumulated in shellfish up to 2.9 µg/mg under laboratory conditions (Grovel, Pouchus & Verbist 2003), and peptaibols were detected from shellfish and sediments from the shellfish farming areas up to 5 ng/g (Poirier, Amiard, et al. 2007; Poirier, Montagu, et al. 2007). Further studies also revealed that shellfish derived fungal strains produced more toxic metabolites, and shellfish extracts enhanced mycotoxin production (Geiger et al. 2013). Using a Diptera larvae bioassay, it was demonstrated that shellfish associated peptaibol enhanced the toxicity of the algal toxin domoic acid by 34.5 times (Ruiz et al. 2010). In Canada, *Trichoderma* fungi are well known from the shellfish growing area of Prince Edward Island (Brewer, Greenwell & Taylor 1993), where an Amnesic Shellfish Poisoning event in 1987 caused 107 cases of human poisoning and 3 deaths (Todd 1993). Shellfish extracts were more potent to neuronal cultures than could be explained from pure domoic acid alone

(Novelli *et al.*, 1992). These studies supported the involvement of mycotoxin in the unexplained shellfish toxicities in 1990s in France, and suggested a possible adverse interaction with co-occurring algal toxins. This emergence of mycotoxin as shellfish toxins is especially concerning because fungal translocation, survival and growth in marine environments are predicted to increase under climate change scenarios.

1.4 Co-occurrence of multiple marine biotoxins

Multiple natural toxins such as algal toxins and mycotoxins often co-occur in aquaculture products and marine environments. Multiple harmful algal bloom (HAB) toxin classes have been reported to co-occur in shellfish and occasionally in mammals from various geographic locations, such as Europe (Campbell et al. 2014), Scotland (Stobo et al. 2008), Australia (Takahashi et al. 2007), New Zealand (MacKenzie et al. 2002), the United States (Peacock et al. 2018) and Chile (García et al. 2015; Zamorano et al. 2013) (Table 1.1). The diarrhetic shellfish poisoning (DSP) toxin okadaic acid often co-occur with other lipophilic toxins such as pectenotoxins and yessotoxins (Alarcan et al. 2018; Peacock et al. 2018). Multiple mycotoxins also co-exist in aquaculture. Several studies detected that aquaculture feeds are often contaminated and co-occurred with several fungal species and mycotoxins including aflatoxins (AF), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB) and ochratoxins (OTA) (Barbosa et al. 2013; Gonçalves et al. 2017; Gonçalves, Naehrer & Santos 2016; Pietsch et al. 2013) (Table 1.2). Some 82% of fish feed samples contained both DON and ZEA (Pietsch et al. 2013), 50% of Brazilian samples contained both AFB_1 and FB_1 (Barbosa et al. 2013), and 50-90 % of samples contained more than one mycotoxin in both European and Asian samples in 2014 and 2015 (Gonçalves et al. 2017). Furthermore, multiple toxigenic fungal species were often isolated from shellfish and the farming areas (Greco et al. 2018; Marrouchi et al. 2013; Zvereva & Vysotskaya 2005) (Table 1.2), suggesting the possibility of co-occurrence of multiple mycotoxins in shellfish.

Table 1.1 Summary of co-occurring major algal toxin groups in shellfish and marine mammals. Paralytic Shellfish Poisoning (PSP), Diarrheic Shellfish Poisoning (DSP) and Amnesic Shellfish Poisoning (ASP).

Co-occurring toxin groups	Location	Species	Reference
PSP/DSP	Chile	mussel (*M.chilensis*), clam (*Venus antiqua*), loco (*Concholepas concholepas*), top shell (*Argobuccinum ranelliforme*)	García et al. (2015)
PSP/ASP	Scotland	king scallop (*Pecten maximus*)	Stobo et al. (2008)
DSP/ASP	United States	bottlenose dolphins (*Tursiops truncatus*)	Fire et al. (2011)
	Australia	oyster (*Saccostrea glomerata,Grassotrea*), mussel (*Modiolus proclivis*), pipis (*Donax deltoides*)	Takahashi et al. (2007)
PSP/DSP/ASP	Europe	mussel (*M.edulis*), scallop (*P.maximus*), oyster (*Crassostrea Gigas*)	Campbell et al. (2014)
PSP/DSP/brevetoxin	United States	manatee (*Trichechus manatus latirostris*), green sea turtle (*Chelonia mydas*)	Capper, Flewelling and Arthur (2013)
PSP/DSP/yessotoxins	Scotland	king scallop (*P.maximus*), mussel (*M.edulis*), queen scallop (*Aequipecten opercularis*)	Stobo et al. (2008)
ASP/DSP/yessotoxin	Scotland	king scallop (*P. maximus*), queen scallops (*A. opercularis*)	Stobo et al. (2008)
	New Zealand	mussel (*Perna canaliculus*)	MacKenzie et al. (2002)
PSP/ASP/DSP/yessotoxin	Scotland	king scallops (*P.maximus*)	Stobo et al. (2008)
PSP/DSP/azaspiracid/yessotoxin	Chile	mussel (*M.chilensis*), clam (*V.antiqua*), loco (*C.concholepas*), top shell (*A.ranelliforme*)	Zamorano et al. (2013)

PSP/DSP/ASP/Microcystin toxins	United States	mussel (*M. californianus*)	Peacock et al. (2018)

Table 1.2 Summary of co-occurring mycotoxins or fungal species in fish feed, shellfish or shellfish farming areas. Underlined percentage represents co-occurring toxins. Percentage and concentrations in brackets indicate occurrence and the level (either average or range) of each mycotoxin in fish feeds, when available. Listed genera were the most commonly detected ones. aflatoxins (AF), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB) and ochratoxins (OTA)

Co-occurring mycotoxin/fungal species	Source	Location	Reference
50%: FB_1(98%, 0.3-4.94 µg/g)/AFB_1(55%)	fish feed		
3.3%: FB_1(98%, 0.3-4.94 µg/g)/AFB_1(55%)/OTA(3.3%) *Cladosporium*(85%)/*Aspergillus*(68%)/*Penicillium*(60%)		Brazil	Barbosa et al. (2013)
82%:DON(82%, 289 µg/kg)/ZEA (100%, 76.2 µg/kg)	fish feed	Europe	Pietsch et al. (2013)
50% (>1 mycotoxin): ZEA(67%, 118.01 µg/kg)/DON(67%, 165.61 µg/kg)/ OTA(67%, 1.53 µg/kg)/FB(30%, 3,419.92 µg/kg)/AF(17%, 0.43 µg/kg)	fish feed	Europe	Gonçalves, Naehrer and Santos (2016)
84% (>1 mycotoxin): AF(68%, 51.83 µg/kg)/ DON(68%, 160.86 µg/kg)/ZEA(58%, 60.41 µg/kg)/ FB(58%, 172.63 µg/kg)/OTA(55%, 2.11 µg/kg)		Asia	
75% (>1 mycotoxin): AF/ZEA/DON/FB/OTA	fish feed	Europe	Goncalves et al. (2017)
90% (>1 mycotoxin): AF(58 µg/kg)/ZEA(53 µg/kg)/DON(29 µg/kg)/FB(58 µg/kg) /OTA		Asia	
Penicillium/*Aspergillus*/*Trichoderma*/*Cladosporium*	shellfish (*Mytilus galloprovincialis*), seawater,sediments	Algeria	Matallah-Boutiba et al. (2012)

Aspergillus/*Penicillium*/*Cladosporium/Chaetomium*	bivalve molluscs (*Crenomytilus grayanus* & *Modiolus modiolus*)	Russia	Zvereva and Vysotskaya (2005)
Aspergillus/*Penicillium*/*Fusarium*/*Trichoderma*	shellfish (*M. galloprovincialis*), sediments, seawater	Italy	Greco et al. (2018)

Despite the frequent co-occurrence of natural toxins, the combined effects of algal toxins and mycotoxins on human health and farmed fish via consumption are largely unknown. Human health impacts of co-occurrence of mycotoxin and algal toxins are even more limited with currently only one published study available (e.g., Ruiz et al. 2010). Broadly, multiple toxin exposure results in additive, synergistic or antagonistic effects (Grenier & Oswald 2011). Broad definitions of additive effects, synergisms and antagonism imply when the combined effects are equal to, greater or less than the sum of individual effects, respectively (Foucquier & Guedj 2015). Currently, a few studies addressed the possible impacts of co-exposure of algal toxins okadaic acid and other lipophilic toxins. *In vivo* mice bioassay studies suggested that co-exposure of okadaic acid and other lipophilic toxins had no combined effect on death rate and pathological changes (Aasen et al. 2011; Aune et al. 2012; Sosa et al. 2013) (Table 1.3). However, *in vitro* study using human intestinal Caco-2 and HICE cells suggested that binary combinations of okadaic acid, yesstoxins and azaspiracid-1 could result in antagonism, additive effect and synergism, depending on the toxin ratio, toxin combination and concentrations (Alarcan et al. 2019; Ferron et al. 2016) (Table 1.3). Furthermore, okadaic acid with yessotoxin and dinophysistoxin 2 displayed enhanced effects, while okadaic acid with 13-desmethyl spirolide C had no combined effect on human neuroblastoma BE(2)-M17 cells (Rodríguez et al. 2015) (Table 1.3). There exist more numerous studies on combined effects of mycotoxins which were comprehensively reviewed by Grenier and Oswald (2011) and Smith et al. (2016). Table 1.4 summarised selected *in vivo* and *in vitro* studies where the combined effects were examined of binary mixtures previously reported in fish feeds (Table 1.4). *In vivo* studies of combined effects have been largely tested on livestock and laboratory animals, and less on aquaculture animals (Gonçalves, Naehrer & Santos 2016) with a few exceptions. Carlson et al. (2001) and McKean et al. (2006) examined the combined effects of aflatoxin B_1 (AFB_1) and fumonisin B_1 (FB_1) on fish. No liver tumour occurrence was observed in rainbow trout fed with only FB1 (3-104 ppm), however those fed with FB1 at 23 and 104 ppm had an increased AFB_1-initiated liver tumour incident rate (Carlson et al. 2001). McKean et al. (2006) observed a strong additive effect on mortality of mosquitofish. DON–ZEN which was previously reported from fish feed (Pietsch et al. 2013) exhibited antagonistic effects on serum and liver tissue metabolic profile of mice (Ji et al. 2017), and delayed hypersensitivity of mice (Pestka et al. 1987). However, co-administration of DON–

ZEN induced significant reduction in splenic resistance to Listeria *monocytogenes* in mice (Pestka et al. 1987) compared to the application of single toxin. In contrast, other reported mycotoxin combinations in fish feeds, the mixture of AFB_1 and FB had synergistic effects on increased in aspartate transaminase, congestion and hemolysis in liver and enlarged thymus of mice, while they showed antagonistic effects on cholesterol, calcium, alkaline phosphatase, triglyceride levels and congestion and hemolysis in spleen (Casado et al. 2001). We currently have no information on carry-over of multiple mycotoxins from feeds to fish (Gonçalves, Naehrer & Santos 2016), however reported synergistic effects from mycotoxin co-exposures suggest possible food safety risks.

Table 1.3 Summary of combined effects of co-occurring HAB toxins. Tested concentration range and ratio are shown in bracket. yessotoxin (YTX), azaspiracid-1 (AZA1), okadaic acid (OA), 13-desmethyl spirolide C (SPX-1), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2), pectenotoxins-2 (PTX-2).

Tested toxin mixture	Tested concentration and/or ratio	Tested animal/cell line	Combined effect(s)	Reference
YTX/AZA1	YTX: 1 or 5 mg/kg AZA1: 200 µg/kg	Mice	• no combined effects on toxin absorption, no combined clinical effect and no combined pathological effect in internal organs	Aasen et al. (2011)
OA/AZA1	OA: 780 or 880 µg/kg AZA1:570 µg/kg	Mice	• no combined pathological effect except toxin mixture showed enhanced absorption in the gastrointestinal tract	Aune et al. (2012)
YTX/OA	YTX: 1 mg/kg OA: 0.185mg/kg	Mice	• no combined effects on mortality, signs of toxicity, diarrhoea and hematological changes	Sosa et al. (2013)
AZA1/YTX	AZA1: 1 YTX: 0.8-3.6	Human intestinal Caco-2 cell	• additive/synergistic effects on cell viability	Ferron et al. (2016)
AZA1/OA	AZA1: 1 OA: 8.2-51	Human intestinal Caco-2 cell	• antagonistic effect on cell viability	Ferron et al. (2016)
YTX/OA	YTX: 1 OA: 4.2-26.5	Human intestinal Caco-2 cell	• antagonistic/additive effects on cell viability	Ferron et al. (2016)

AZA1/YTX	AZA1: 1 YTX: 0.8-3.6	Human intestinal HIEC cell	• additive/synergistic effects on cell viability	Ferron et al. (2016)
AZA1/OA	AZA1: 1 OA: 8.2-51	Human intestinal HIEC cell	• additive/ antagonistic effects on cell viability	Ferron et al. (2016)
YTX/OA	YTX: 1 OA: 2-26.5	Human intestinal HIEC cell	• additive/synergistic/antagonistic effects on cell viability	Ferron et al. (2016)
OA/SPX-1	OA: 1-200 nM SPX-1: 50 nM	Human neuroblastoma BE(2)-M17 cell	• no combined effect on cell viability	Rodríguez et al. (2015)
OA/YTX	OA: 1-500 nM YTX: 500 nM	Human neuroblastoma BE(2)-M17 cell	• mixture enhanced decrease in cell proliferation, but no combined effect on cell viability	Rodríguez et al. (2015)
OA/DTX2	OA: 1-200 nM	Human neuroblastoma BE(2)-M17 cell	• mixture enhanced decrease in cell viability	Rodríguez et al. (2015)

	DTX2: 100 nM			
OA/PTX-2	OA: 18.75-600 nM PTX-2: 6.25-200 nM OA:PTX-2 =3:1	Human intestinal Caco-2 cell	• additive/antagonistic/synergistic effects on cell viability • additive/antagonistic effect on ROS (reactive oxygen species) production • antagonistic/additive effects on γ-H2AX phosphorylation • antagonistic/synergistic/additive effects on IL-8 (interleukin 8) release	Alarcan et al. (2019)
OA/SPX-1	OA: 18.75-600 nM SPX-1: 2.1-66.7 nM OA:SPX-1 = 8.92:1	Human intestinal Caco-2 cell	• additive/synergistic effects on viability • antagonisms/additivity on ROS production and γ-H2AX phosphorylation	Alarcan et al. (2019)
OA/YTX	OA: 18.75-600 nM YTX: 6.25-200 nM OA:YTX= 3:1	Human intestinal Caco-2 cell	• additive/synergistic effect on viability • antagonisms/additivity on ROS measurement and γ-H2AX phosphorylation • antagonisms/synergism on IL-8 release	Alarcan et al. (2019)

Table 1.4 Summary of combined effects of two reported co-occurring mycotoxins in fish feed, shellfish or shellfish farming areas. Combined effects of both *in vivo* and *in vitro* are presented when the information is available. Aflatoxin B1 (AFB1), zearalenone (ZEA), deoxynivalenol (DON), fumonisin B1 (FB1) and ochratoxins (OTA).

Tested mycotoxin mixture	Tested concentration and/or ratio	Tested animal/cell line	Combined effect	Reference
AFB1/ZEA	AFB1: 50µg/kg dry matter ZEA: 500µg/kg dry matter	Laoshan dairy goats	• no significant effects on dry matter intake, milk yield, body mass, average daily gain, hematological and serum biochemical parameters	Huang et al. (2018)
AFB1/ZEA	AFB1: 1 nM or 100 nM ZEA: 10^{-12} to 10^{-6} M	Human breast cancer MCF-7 cell	• significant interactive effects on cell growth and cell cycle. AFB1 reduced the cytotoxicity effect caused by ZEA.	Yip et al. (2017)
AFB1/FB1	AFB1: 1.0×10^{-2} mg/g FB: 1.0×10^{-5} mg/g	Swiss mice	• enhanced increases in the enzymatic activity of aspartate transaminase, congestion and haemolysis, enlarged thymus and intensified lesions, compared to the single dosed groups. • weakened decreases in serum cholesterol, triglyceride, alkaline phosphatase, calcium levels compared to the single dosed group.	Casado et al. (2001)
AFB1/FB1	AFB1: 0.13-1.00 µM FB1: 49.9-399.2 µM	Human liver cancer HepG2 cells	• no congestion and haemolysis in spleen was observed while these were present in the group treated with FB1 only. • weak antagonism on cytotoxicity	Mckean et al. (2006)

DON/ZEA	DON: 5 ppm ZEA: 10 ppm	Female B6C3F1 mice	• no combined effects on weight gain, organ weight, histological, haematological profile, and serum immunoglobulin levels	Forsell et al. (1986)
DON/ZEA	DON: 10 or 20 µM ZEA: 10 or 20 µM	Human intestinal Caco-2 cells	• nearly additive effects on cell viability and inhibiting cellular protein synthesis • synergistic effects on lipid peroxidation	Kouadio et al. (2007)
AFB1/DON	AFB1: 2.5 mg/kg bodyweight (BW) DON: 5.0 mg/kg bodyweight (BW)	weanling BALB/c female mice	• synergistic effects on increase in activity of the alanine aminotransferase (ALT), the hepatic malondialdehyde (MDA) content, mRNA level of caspase-3 and decrease in mRNA level of B-cell lymphoma 2 (Bcl-2)	Sun et al. (2014)
AFB1/DON	AFB1: 0.01-0.02 µg/ml DON: 0.25-0.5 µg/ml	Primary hepatocytes of carp (*Cyprinus carpio*)	• enhanced inhibition of cell growth, cell structure destruction, and increase in activity of aspartate aminotransferase (AST), ALT and lactate dehydrogenase (LDH) compared to the single dose.	He et al. (2010)
AFB1/OTA	AFB1: 0.25 mg/kg BW OTA: 0.5 mg/kg BW	Young male F344 rats	• reduced decrease in the ALT level, the bone marrow micronucleus (MN) induction compared to the AFB1 single administration. • significantly increase net formamidopyrimidine DNA glycosylase (FPG)-sensitive sites in kidney and liver	Corcuera et al. (2015)
AFB1/OTA	AFB1: 5-50 or 1-20 µM	Monkey kidney vero cells	• additive effects on cytotoxicity	Golli-Bennour (2010)

	OTA: 5-50 µM, or fixed 1µM		• enhanced increase in DNA fragmentation and P53 level, and downregulated bcl-2 expression, compared to single dose	
ZEA/FB1	ZEA: 15 g/day FB1: 150 µg/day	adult, male Wistar Crl:WI BR rats	• no combined effect on liver, kidney and spleen weights, hepatic phospholid fatty acid composition • increased the reduced glutathione concentration (GSH) • synergisitic effect on lowering the unsaturation index (UI) in the hepatic phospholipid fatty acid profile • antagonistic effect on the stearic acid proportion and glutathione peroxidase activity •	Szabo et al. (2018)
ZEA/FB1	ZEA: 5,10,20 µM FB1: 10 µM	Human intestinal cell line Caco-2	• less than additive effect on cytotoxicity • more than additive effects on malonedialdehyde (MDA) increases • more effective in cellular protein inhibition while less effective on DNA synthesis inhibition, compared to the single dose	Kouadio et al. (2007)
ZEA/OTA	ZEA: 1.12-41.28 µM OTA: 6.61-37.30 µM	Human cancer cell line HepG2 Cellosaurus cell line KK-1	• additive effects on cell viability and antagonistic effect at lower concentration. • No significant additive effect on reactive oxygen species formation • synergistic effects at higher concentration on reactive oxygen species formation	Li et al. (2014)
DON/FB1	DON: 30 µg/day FB1: 150 µg/day	male Wistar Crl:WI BR rats	• no significant effect on liver weight • additive effect on the proportion of arachidic acid. • significant increase in the proportion of myristic acid, compared to the control group.	Szabo et al. (2018)

			• antagonistic effects on the glutathione peroxidase activity	
DON/FB1	DON: 4, 10 and 20 µM FB1: 10 µM	Human epihelial cell Caco-2	• more strongly reduced cell viability than either DON or FB1 toxin alone • synergistic effects on increase MDA production • additive effects on protein synthesis • less than additive effects on DNA synthesis	Kouadio et al. (2007)
DON/OTA	DON: 25µg/kg BW OTA: 18ug/kg BW	weaner pigs	• significantly increased leukocytes counts compared to the control • significantly increased phagocytosis and apoptosis, but not as much as the response to OTA alone • radical formation was inhibited in a combination, but promoted with single OTA treatment • keyhole limpet haemocyanin (KLH) was significantly suppressed in only combination of DON and OTA	Muller et al (1999)
DON/OTA	DON: 0.5 – 30 µM OTA: 5 - 80 µM	Human epithelial cell Caco-2	• binary mixture at non-toxic concentrations (when toxins are treated individually) of 0.5 µM DON and 20 µM OTA caused a significant increase in cytotoxicity • toxicity caused by DON was significantly increase by increasing doses of OTA (40 and 80 µM)	Cano-Sancho et al. (2015)
OTA/FB1	OTA: 3 mg/kg of diet FB1: 300 mg/kg of diet	Turkey Poults	• binary mixture caused the lowest body weight gains compared to other treatment types including single dose and control. • only mixture caused increases in serum concentration of uric acid and creatinine, activity of alanine transferase.	Kubena et al (1997)

OTA/FB1	OTA: 10 µM FB1: 5, 10, 25, 50 µM	Rat brain glioma C6, Caco-2 and Vero cells	• Synergistic effects on reduction of cell viability	Creppy et al. (2004)

1.5 Analytical methods for assessing combined toxicity

Characterization of combined effects of toxins and drugs have been a challenging problem which results in the development of several methodological models (Foucquier & Guedj 2015). The main approaches to assess the combined effects include effect-based approaches and dose-effect-based approaches (reviewed in Foucquier & Guedj 2015). Approaches deviated from effect-based strategy examined the effects from two drug treatments, compared to the effects from a single drug treatment (Foucquier & Guedj 2015). Conclusions fall into either positive, negative or no combined effects (Foucquier & Guedj 2015). Typical approaches based on effect-based strategy are Combination Subthresholding, Highest Single Agent, Response Additivity and Bliss Independence models (Foucquier & Guedj 2015). Limitations of these effect-based strategies are, for example, that commonly used Bliss Independence model assume that two toxins act independently (Roell, Reif & Motsinger-Reif 2017). However, most cases of toxin interactions do not meet this assumption (Gessner 1988). In contrast, dose-effect-based approaches compare nonlinear dose-effect curves of single and combined treatments, include models such as Combination index and isobologram analysis (Berenbaum 1977; Foucquier & Guedj 2015). This approach is based on Loewe additivity models, and provide definite conclusions of synergistic, additive and antagonistic effects (Foucquier & Guedj 2015). Limitations of dose-effect-based approaches are that it requires accurate estimation of dose-effect curves (Foucquier & Guedj 2015), and the constant potency ratio (Grabovsky & Tallarida 2004). These requirements result in expensive experiments and computations, constant potency is regarded as rare in practice and non-constant potency ratio leads to more technical analysis (Foucquier & Guedj 2015; Grabovsky & Tallarida 2004).

1.6 Shortcomings of current shellfish regulations

Current shellfish safety regulations do not account for the risk management of co-occurring toxins and emerging toxins. The current regulatory limits for algal toxins and mycotoxins are established based on individual toxicity by single compounds, and take no account of combined effects (Smith et al. 2016; Wekell, Jurst & Lefebvre 2004). Similarly, the regulatory limits for mycotoxins have been developed based on the individual toxicity. Table 1.5 summarises regulatory limits of major algal toxins and mycotoxin (Commission Regulation 2006; EFSA Panel on Contaminants in the Food Chain 2009). This current regulatory setting is problematic in view of limited knowledge on combined effects of natural toxins. Furthermore, the majority of the current shellfish monitoring depend on chemical analytical methods such as high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS) as alternative methods to mouse bioassays (Campbell et al. 2011). Analytical methods detect only the target toxins and are unable to detect emerging toxins such as mycotoxins, nor provide estimates of total toxicity as provided by mouse bioassays (Botana et al. 2016). Furthermore, the combined effects of co-occurring toxins often cannot be predicted from the mode of action of individual toxins (Chou 2006). The current regulatory approaches could fail when there are increasing risks of emerging mycotoxins and occurrence of multiple toxins. This emphasizes the importance of studies assessing effects of multiple toxin exposure to incorporate the combined effects into regulatory limits in conjunction with chemical detection methods.

Table 1.5 Summary of regulatory limits for algal toxins and mycotoxins set by the European Commission (EC).

Toxin Group	Limits in shellfish meat/foodstuffs
Algal toxins	
Okadaic acid (OA) and analogues[1]	160 µg OA equivalents/kg
Azaspiracid (AZA)	160 µg AZA equivalents/kg[2]
Pectenotoxins (PTX)	160 µg OA equivalents/kg[3]
Yessotoxin (YTX)	1 mg YTX equivalents/kg[4]
Saxitoxin (STX)	800 µg PSP/kg
Domoic acid (DA)	20 mg DA/kg
Mycotoxins	
Aflatoxin B1	0.1-12 µg/kg
Zearalenone	20-400 µg/kg
Deoxynivalenol	200-1,750 µg/kg
Fumonisin B1 and B2	200-4,000 µg/kg
Ochratoxin A	0.5-80 µg/kg

[1]Analogues include dinophysistoxins and PTX; [2]AZA equivalents refer to AZA1, AZA2 and AZA3; [3] Pectenotoxins shares the same limits for OA equivalents; [4]YTX equivalents include YTX, 1a-homoYTX, 45-hydroxyYTX and 45-hydroxy-1a-homoYTX.

1.7 Research objectives

Fungi are emerging pathogens to marine organisms and as contaminants in seafood. Their impacts on marine ecosystems and seafood are predicted to increase due to climate change. Especially in Australia, dust storms are predicted to increase, and the dust storm associated *A. sydowii* bloom in 2009 in coastal areas raised the alarm about possible marine ecosystem and human health impacts (Hallegraeff et al. 2014). However, our current knowledge on fungi in marine environments is limited, compared to terrestrial fungi. Their broad impacts on marine animals remain largely unknown. *A. sydowii* has previously caused significant mortality loss in the Caribbean, however previous studies have not fully explored the role of *A. sydowii* secondary metabolites in sea fan aspergillosis disease, and disease impacts on the coral endosymbiont *Symbiodinium*. Additionally, the impacts of mycotoxins

on human health through contaminated seafood consumption especially with co-occurring algal toxins in shellfish, has remained unclear.

To achieve those objectives, the following broad aims were addressed.

i) Elucidation of pathogenicity of *A. sydowii* as the cause of sea fan coral aspergillosis, focusing on differences in metabolite profiles between pathogenic and non-pathogenic strains, and their impacts on the coral endosymbiont *Symbiodinium* **[Chapter 2]**.

ii) Assessment of broader impacts of emerging mycotoxins on marine organisms using a fish gill cell line model **[Chapter 3]**.

iii) Assessment of combined toxicity of mycotoxins and phycotoxins on human cell line models **[Chapter 4]**.

Chapter 2. *Aspergillus sydowii* marine fungal bloom in Australian coastal waters, its metabolites and potential impact on *Symbiodinium* dinoflagellates*

Abstract

Dust has been widely recognised as an important source of nutrients in the marine environment and as a vector for transporting pathogenic microorganisms. Disturbingly, in the wake of a dust storm event along the eastern Australian coast line in 2009, the Continuous Plankton Recorder collected masses of fungal spores and mycelia (~150,000 spores/m^3) forming a floating raft that covered a coastal area equivalent to 25 times the surface of England. Cultured *A. sydowii* strains exhibited varying metabolite profiles, but all produced sydonic acid, a chemotaxonomic marker for *A. sydowii*. The Australian marine fungal strains share major metabolites and display comparable metabolic diversity to Australian terrestrial strains and to strains pathogenic to Caribbean coral. Secondary colonisation of the rafts by other fungi, including strains of *Cladosporium*, *Penicillium* and other *Aspergillus* species with distinct secondary metabolite profiles, was also encountered. Our bioassays revealed that the dust-derived marine fungal extracts and known *A. sydowii* metabolites such as sydowic acid, sydowinol and sydowinin A adversely affect photophysiological performance (F_v/F_m) of the coral reef dinoflagellate endosymbiont *Symbiodinium*. Different *Symbiodinium*

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clades exhibited varying sensitivities, mimicking sensitivity to coral bleaching phenomena. The detection of such large amounts of *A. sydowii* following this dust storm event has potential implications for the health of coral environments such as the Great Barrier Reef.

2.1 Introduction

In a previous publication (Hallegraeff et al. 2014), we reported an extensive *Aspergillus sydowii* marine fungal bloom in the wake of an Australian dust storm event in 2009. The Continuous Plankton Recorder (CPR) collected masses of fungal spores and mycelia, estimated to be up to 150,000 spores per m^3, between Brisbane and Sydney, an area equivalent to 25 times the surface of England. Fungal spores and mycelia were identified as *Aspergillus sydowii* using molecular sequencing of three different genes (large-subunit rRNA gene, internal transcribed spacer and beta tubulin) with 99%–100% match. *A. sydowii* has been widely claimed to cause aspergillosis in Caribbean gorgonian corals.

Aspergillus species are pathogenic to a wide range of organisms (Burge et al. 2013). In marine environments, *A. sydowii* is characterised as a causative agent of aspergillosis of sea fan corals, based on morphological, physiological and nucleotide sequence analysis, and Koch's postulate (Kim & Harvell 2004; Smith et al. 1996). *A. sydowii* is essentially a terrestrial organism, unable to sporulate and complete its life cycle in seawater (Smith et al. 1996). Symptoms of aspergillosis include small lesions of necrotic tissue with purple halos (Smith et al. 1996), resembling the pathology of coral bleaching and hence suggesting an impact on *Symbiodinium* dinoflagellate symbionts. This fungal species is known to infect several species of octocorals (Smith & Weil 2004), and has caused 20%–90% sea fan mortality in the Florida Keys (Bruno et al. 2011). Due to the significant mortality and subsequent changes in the coral community structure, research focus needs to shift from the etiology of the disease to greater understanding of the interactions among causal pathogen, coral and their endosymbionts *Symbiodinium* (Correa et al. 2009).

A putative virulence factor was initially proposed for *A. sydowii* strains isolated from diseased sea fan corals, although molecular genetic analysis reveals no clear differences between pathogenic and non-pathogenic strains (Geiser et al. 1998; Rypien, Andras &

Harvell 2008). Furthermore, there were no clear differences in temperature tolerance, susceptibility of coral host crude extract and carbon source utilization patterns (Alker, Smith & Kim 2001). However, Geiser et al. (1998) found that when sea fans were inoculated with virulent isolates from affected sea fans, all showed typical symptoms of aspergillosis at the point of inoculation, whereas sea fans with avirulent isolates showed no symptoms.

The role of secondary metabolites in pathogenesis has been largely unexplored (Smith & Weil 2004). While over thirty metabolites from *A. sydowii* have been reported in the literature, most exhibit chemistry related to sydonic acid and sydowinin (Hamasaki, Nagayama & Hatsuda 1978; Hamasaki, Sato & Hatsuda 1975a; Li et al. 2015; Liu et al. 2013; Trisuwan et al. 2011). Comparative HPLC analysis of marine pathogenic and non-pathogenic *A. sydowii* strains demonstrate overlapping metabolite profiles, but none were attributable to specific *A. sydowii* metabolites (Malmstrøm et al. 2001). Therefore, the composition of the metabolite profiles and their relationship to pathogenesis remain unclear.

The aim of this study was to examine metabolic profiles of new fungal isolates from the 2009 Australian dust storm plankton silks and compare their metabolic profiles with those from other sources including terrestrial habitats and diseased Caribbean sea fan corals. We also re-evaluate the fungal diversity in the 2009 plankton rafts, and assess the impacts of *A. sydowii* metabolites on various strains of the dinoflagellate coral endosymbiont, *Symbiodinium*.

2.2 Materials and methods

2.2.1 Fungal isolation from the Continuous Plankton Recorder silks and *A. sydowii* strains

Fungi were isolated from the formalin-preserved Continuous Plankton Recorder (CPR) silks by scraping spores from the silks and inoculating them on MEA, or producing aqueous spore suspensions as follows. Approximately 5 × 5 cm of the CPR silk samples were added to 2 mL of autoclaved sterile Milli-Q water and vortexed for 20 s to remove embedded spores and mycelium from the plankton silk. The suspension was centrifuged at 10,000× *g*

for 5 min. Supernatants were removed and the pellet re-suspended in 2 mL of Milli-Q water. Spore suspensions or spores floating on top of Milli-Q water were inoculated on either full strength, or half strength MEA with tetracycline (40 µg/mL) using a 10-µL disposal-inoculating loop. Morphologically different fungal colonies were selected and their metabolite profiles analyzed by HPLC.

A. sydowii strains from non-marine environments were supplied by Dr. John Pitt and Dr. Ailsa Hocking, Commonwealth Scientific and Industrial Research Organisation (CSIRO) Food Research North Ryde (FRR) Culture Collection (Appendix, Table A.1). Two *A. sydowii* strains isolated from diseased sea fan corals (FK1) were supplied by Prof. Drew Harvell, Cornell University, USA.

2.2.2 HPLC analysis on fungal secondary metabolites

Fungal cultures from the CPR silk after the dust storm in 2009 and five strains from CSIRO were grown in a wide range of solid media. The agars, Czapek-dox agar (CZA; 30 g Sucrose, 3 g Sodium nitrate, 1 g Di-potassium phosphate, 0.5 g Magnesium sulphate, 0.5 g Potassium chloride, 0.01 g iron(II) Sulphate heptahydrate, 15 g Agar, 1L distilled water), malt extract agar (MEA; 20 g Malt extract, 20 g Glucose, 1 g Peptone, 20 g Agar, 1 L distilled water), yeast extract and sucrose agar (YES; 20 g Yeast extract, 150 g Sucrose, 20 g Agar, 1 L distilled water), and glycerol casein agar (GCA; 10 g Glycerol, 0.3 g Casein, 0.3 g Potassium nitrate, 2 g Sodium chloride, 2 g Dipotassium phosphate, 0.05 g Magnesum sulfate heptahydrate, 0.02 g Calcium carbonate, 0.01 g Iron(II) sulphate heptahydrate, 18 g agar, 1 L distilled water) were prepared. The grains, barley, rice (jasmine and basmati) and cracked wheat were prepared by hydration (~30 g with 30 mL water in a 250 mL flask) during sterilisation (120 °C for 40 min.). The agar and grains were inoculated with a suspension of fungal spores and incubated at 24 °C and sampled at seven and 14 days. Subsamples (1 g) of the cultures were extracted with methanol (2 mL) for a minimum of 1 h on a wrist shaker, centrifuged (15,700 × *g* for 3 min., Eppendorf) and analysed by HPLC. Small sections of silk material (locations 2 and 3) were also directly extracted with methanol and processed by HPLC.

Analytical HPLC was performed on a gradient Shimadzu HPLC system comprising an LC-10AT VP gradient chromatograph, SPD-M10A VP diode array detector and SCL-10A VP system controller. The column used was an Alltima C_{18} rocket format column (100 Å, 53 × 7 mm, 3 µm; Grace Discovery, Deerfield, IL, USA) eluted with a 3 mL/min gradient of 10%–100% MeCN/H_2O (+0.01% TFA) over 7 min. The HPLC traces were accessioned into our in-house database, COMET (Lacey & Tennant 2003) and the major metabolites were analysed by retention time and UV-Vis spectrum fit against a library of known metabolites (>25,000 spectra) and type species library (>25,000 spectra from 2000 fungal species).

HPLC-DAD-ESI(±)MS was performed on an Agilent 1260 UHPLC coupled to an Agilent 6130B single quadrupole mass spectrometer. The column was an Agilent Zorbax Rapid Resolution HT Eclipse Plus C_{18} (50 × 2.1 mm, 1.8 µm) eluted with a 0.5 mL/min gradient of 10%–100% MeCN/H_2O (+0.025% formic acid) over 10 min.

2.2.3 *Symbiodinium* dinoflagellate strains

Strains of the endosymbiotic dinoflagellate *Symbiodinium* were obtained from the Australian National Algal Culture Collection (ANACC) in Hobart. Strains CS73, CS156 and CS163 were selected based on genetic clades, growth rate and geographic origin (Table 2.1). Strains were grown in f2 media (Guillard & Ryther 1962) and maintained at 25 °C under 12/12 h light/dark cycle. Algal cell counts were undertaken using a haemocytometer.

Table 2.1 *Symbiodinium* dinoflagellate strains/clades used in the bioassay.

CS-No.	Clade	Source Location
CS-73	Clade A	Heron Is., Great Barrier Reef, Queensland, Australia
CS-156	Clade C	Hawaii, USA
CS-163	Clade A1	Palau

2.2.4 Crude extracts and typical *A. sydowii* secondary metabolites

Four typical *A. sydowii* metabolite standards sydowinol, sydowinin A, sydowinin B and sydowic acid were provided by Professor Hiromitsu Nakajima, Tottori University, Japan. Crystallized metabolites were dissolved in ≥99.9% acetone and diluted to 70% with autoclaved sterile Milli-Q water. Known weights of methanol evaporated crude extracts of day 7 optimisation of FRR 5152 and ASBS were dissolved in 2.6% methanol and sonicated to maximize solubility of metabolites. Crude extracts were then filter-sterilized (Millex GP 0.22 μm).

2.2.5 *Symbiodinium* photophysiology assays

Either 0.01 mg or 0.1 mg of standard *A. sydowii* metabolites, or 0.1 mg or 0.3 mg of FRR5152 or ASBS extracts were added to 24 well microplates (Greiner Bio-one, Frickenhausen, Germany). Solvents were completely evaporated in sterile conditions prior to the addition of 1.0×10^5 cells/mL exponential growth stage *Symbiodinium* dinoflagellate cell culture. The maximum quantum yield (F_v/F_m) of *Symbiodinium* was measured from the bottom of each well using an underwater pulse amplitude modulated fluorometer (Diving-PAM, Walz, Effeltrich, Germany). Algal cultures were dark adapted for 30 to 60 min at 25 °C before each measurement. Instrument gains were adjusted between 1 and 12, and F_0 (background chlorophyll fluorescence) set to the range of 200 to 400. This assay was conducted in triplicate, and average values of three measurements of each replicate were taken. Samples were dark adapted again for second/third measurements. Day 0 indicates before adding the fungal metabolites. This assay was conducted for 8 days, and measurements were taken every second day to follow the impact of fungal metabolites on the dinoflagellate photosynthetic performance.

2.2.6 Statistical analysis

Statistical analyses were performed using R (R Core Team, 2017). One-way analysis of variance (ANOVA) was conducted to test significant differences in maximum quantum yield among treatments each day. When the main effect was significant, Tukey’s honestly

significant difference (HSD) *post hoc* tests were conducted. Box–Cox transformation was applied to determine appropriate transformation to improve normality and homogeneity of variance. A significance level of 0.05 was applied.

2.3 Results

2.3.1 Australian terrestrial *A. sydowii* strains

The metabolite consensus for *A. sydowii* was developed by examining seven strains held in the Food Research North Ryde (FRR) Fungal Culture Collection. Five of the strains were sourced from Australian terrestrial locations and two from Indonesian terrestrial locations. Cultivation of the strains in liquid and on agar gave low levels of secondary metabolites, with yeast extract and sucrose (YES) agar consistently superior in promoting abundance and diversity, consistent with previous reports (Malmstrøm et al. 2001). Further increases in production (5- to 10-fold) were achieved by cultivation on grains, in particular, rice. Strains showed some variation, but overall gave a consistent profile of major metabolites (Appendix, Figure A.1). Strain FRR5068 was considered representative of *A. sydowii* and the co-metabolite profile of this species *in sensu*. Metabolites from *A. sydowii* (FRR5068) grown on rice for 14 days and extracted with methanol were separated by gradient HPLC using diode array detection (DAD) and mass spectrometry (MS) (Figure 2.1A). Assessment of the co-metabolite diversity was undertaken using UV detection at 210 nm, with 78 discrete secondary metabolites being responsible for 99.5% of the total area under metabolite peaks (AUC) from 0.5 to 10.5 min. Analysis of the percent abundance of the metabolites revealed a hyper-dispersed distribution, with most metabolites present in trace amounts (Appendix, Table A.2) and only 25 metabolites responsible for 90% of total metabolite AUC. The metabolite distribution can be described in terms of the metabolite's polarity on reverse phase (C_{18}) HPLC. Four unidentified polar metabolites eluting 0.5–1.0 min accounted for 8.9% of the total metabolite AUC. These compounds exhibited simple UV-Vis spectra (λ_{max} 200–210 nm), but did not ionize in the mass spectrometer, precluding identification. The intermediate-eluting peaks from three to seven minutes contained a complex series of metabolites dominated by variants of three distinct UV-Vis spectral classes (Figure 2.2). Comparison of

the retention times, UV-Vis spectra and MS data of a mixture of known *A. sydowii* metabolites (Figure 2.1, Standards) identified five of the six major metabolites as sydowinol (t_R 3.44 min, 0.51%; λ_{max} 212, 268, 301, 343 nm; ESIMS *m*/*z* $[M - H]^-$ 317, $[M + H]^+$ 319), hydroxysydonic acid (t_R 3.85 min, 16.3%; λ_{max} 213, 248, 303 nm; ESIMS *m*/*z* $[M - H]^-$ 281, $[M + Na]^+$ 305), sydowinin B (t_R 4.27 min, 6.9%; λ_{max} 237, 265, 297, 390 nm; ESIMS *m*/*z* $[M - H]^-$ 315, $[M + H]^+$ 317), sydowinin A (t_R 5.16 min, 10.8%; λ_{max} 206, 234, 258, 300, 370 nm; ESIMS *m*/*z* $[M - H]^-$ 299, $[M + H]^+$ 301), sydonol (t_R 6.15 min, 1.6%; λ_{max} 202, 219, 280 nm; ESIMS *m*/*z* $[M - H]^-$ 251) and sydonic acid (t_R 6.33 min, 16.0%; λ_{max} 215, 248, 304 nm; ESIMS *m*/*z* $[M - H]^-$ 265, $[M + Na]^+$ 289). The sixth metabolite, eluting at 4.01 min and constituting 9.1% of the metabolite AUC, exhibited a sydonic acid UV-Vis spectrum and molecular weight 282 amu, and is tentatively considered an isomer of hydroxysydonic acid. The known metabolites constitute 50.4% of the total metabolite AUC. The remaining eight major metabolites could be identified by their respective UV-Vis spectra as analogues of sydonic acid, sydowinin or sydonol, while six metabolites could not be assigned to a specific chemical based on their UV-Vis spectra. The non-polar region from 7 to 10.5 min contained two metabolites, a sydowinin analogue (7.30 min, 1.22%) and a sydonol analogue (9.17 min, 0.88%), with the final metabolite, a fatty acid (λ_{max} < 200 nm) hydrolysed from grain oils (10.30 min, 4.04%).

2.3.2 Australian marine *A. sydowii* ASBS strain

The *A. sydowii* ASBS strain was isolated by towing a Continuous Plankton Recorder (CPR) instrument through a microbial raft located at 28.424°S, 153.811°E off the Australian mainland after the 2009 dust storm (Hallegraeff et al. 2014). Genetic sequences of spores from the CPR silks indicated the strain to be a 99%–100% match to *A. sydowii* (Hallegraeff et al. 2014). The metabolite profile of *A. sydowii* ASBS grown on rice for 14 days and extracted with methanol is presented in Figure 1B. Visual inspection of Figure 2.1A, B show a strong overlap in the both the polar and intermediate polar regions of the HPLC traces. Assessment of the co-metabolite diversity revealed 63 metabolites were responsible for 99.5% of the total metabolite AUC of the HPLC trace from 0.5 to 10.5 min. Like FRR5068, the metabolite distribution was hyper-dispersed, with most metabolites present in trace amounts and only 13

metabolites responsible for 90% of the total metabolite AUC (Appendix, Table A.3). More than half (57.3%) of the co-metabolite distribution comprised five of the known *A. sydowii* metabolites: hydroxysydonic acid (3.85 min, 13.8%), sydowinin B (4.27 min, 11.6%), sydowinin A (5.16 min, 8.0%), sydonol (6.15 min, 1.1%) and sydonic acid (6.32 min, 24.0%). Retention times and UV-Vis spectra of each metabolite were consistent with the authentic standards.

2.3.3 US marine *A. sydowii* FK1 strain

The *A. sydowii* FK1 strain was identified as the causal pathogen of *Gorgonia ventalina* from Key West, Florida, USA. The metabolite profile of *A. sydowii* FK1 grown on rice for 14 days and extracted with methanol exhibited less metabolic diversity than the Australian *A. sydowii* cultures (Figure 2.1C), with only 48 metabolites being responsible for 99.5% of the total metabolite AUC and 17 metabolites responsible for 90% of the AUC (Appendix, Table A.4). The HPLC was nonetheless dominated by the known metabolites with sydowinol (3.44 min, 8.5%), hydroxysydonic acid (3.86 min, 6.1%), sydowinin B (4.27 min, 18.6%), sydowinin A (5.16 min, 1.1%), sydonol (6.15 min, 0.92%) and sydonic acid (6.32 min, 20.8%) constituting 55% of the metabolite AUC. The relative abundance of the known metabolites is different from the Australian strains, with increased levels of sydowinol and sydowinin B at the expense of hydroxysydonic acid and sydowinin A. The strain also displays high abundance of unidentified metabolites not observed in the Australian strains, eluting from 2.95 to 3.73 min. The six metabolites constitute nearly 17% of the co-metabolite profile and their respective retention times (λ_{max} and *m*/*z*) are a chlorinated acidic metabolite eluting at 2.95 min (λ_{max} 198, 210 s, 268, 300 s nm, ESIMS *m/z* $[M - H]^-$ 377/379) together with a dichloro analogue eluting at 3.62 min (λ_{max} 198, 220 s, 270, 330 s nm, ESIMS *m*/*z* $[M - H]^-$ 411/413), three polar neutral metabolites eluting at 3.08 min (λ_{max} 198, 220, 265 nm, ESIMS *m/z* $[M - H]^-$ 230, $[M + H]^+$ 232), 3.28 min (λ_{max} 212, 226 s, 242 s, 298 s, 313 s, 331 nm, ESIMS *m/z* $[M - H]^-$ 235, $[M + H]^+$ 237) and 3.36 min (λ_{max} 192, 225 s, 240, 258 s, 328 nm, ESIMS *m/z* $[M - H]^-$ 235, $[M + H]^+$ 237). A final metabolite eluting as a broad peak at 3.73 min failed to provide useable MS ionisation, but possessed a highly characteristic UV spectrum (λ_{max} 230, 254, 278 s, 333 nm). The metabolites appear unique among *A. sydowii*

strains. Further, the λ_{max} values are inconsistent with those for the *A. sydowii* co-metabolites described in the literature. The list of the *A. sydowii* metabolites described in the literature is provided in Table 2.2

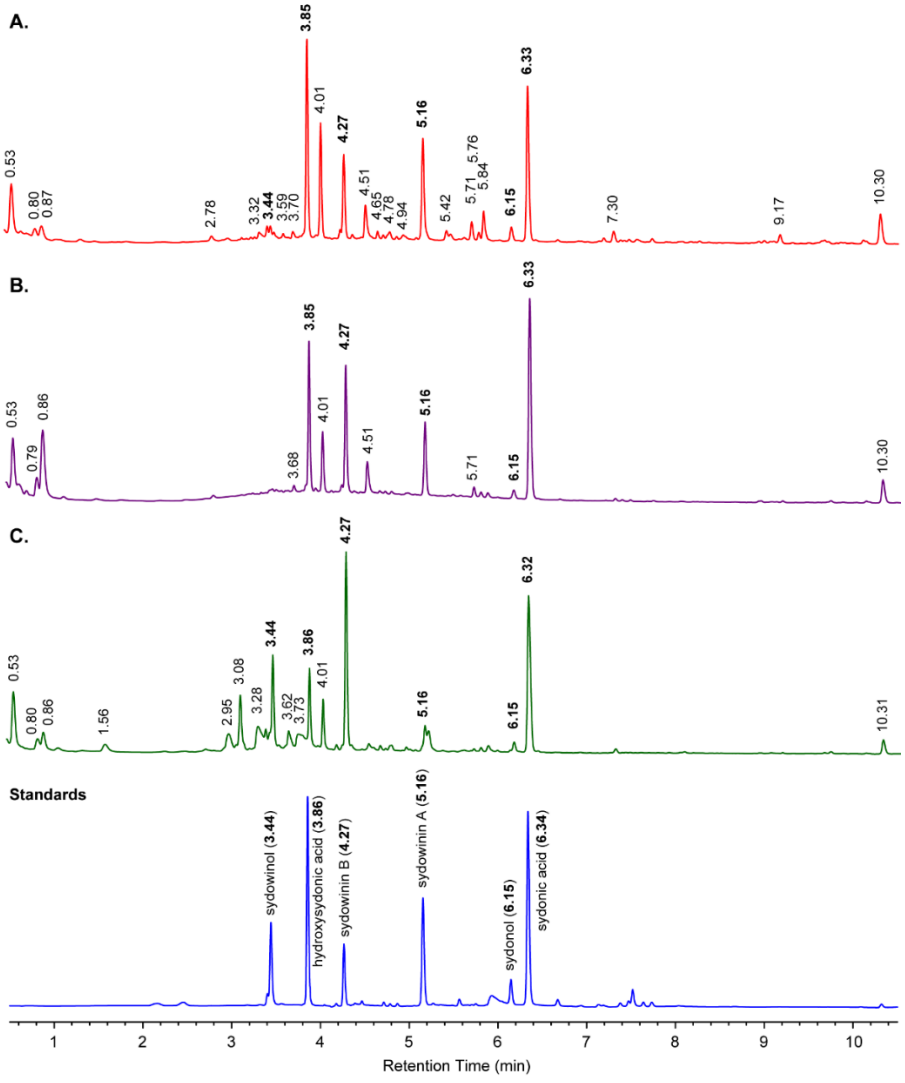


Figure 2.1 Comparison of HPLC traces (210 nm) of secondary metabolites of methanolic extracts from selected strains of *A. sydowii* grown on rice: (A) Terrestrial strain FRR5068; (B) Marine strain ASBS; (C) US Marine pathogenic strain FK1; Standards: metabolites isolated from *A. sydowii* and maintained in MST's metabolite library. The HPLC trace has been truncated from 0.5 to 10.5 min to remove polar endogenous and primary metabolites (<0.5 min) in the solvent front and non-polar grain oils (>10.5 min.).

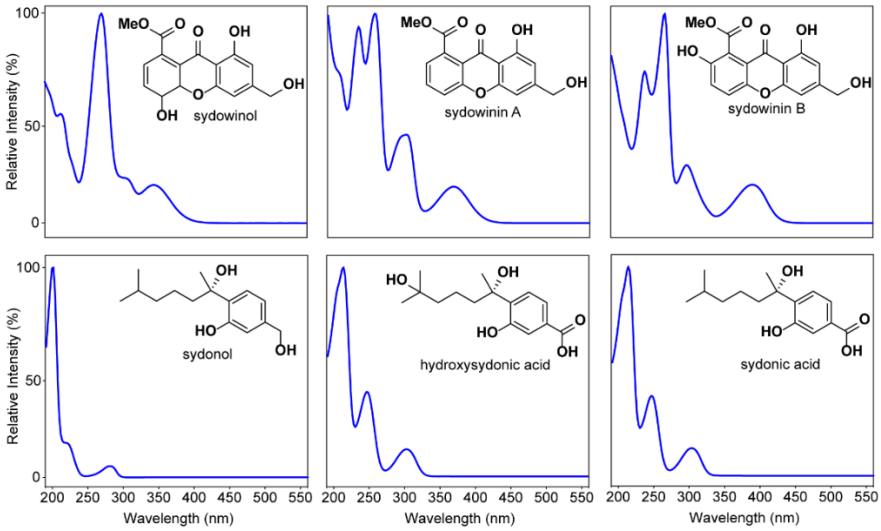


Figure 2.2 The structures and UV-Vis spectra of *A. sydowii* metabolite standards.

Table 2.2 Metabolites previously isolated from *A. sydowii*.

A. sydowii metabolites	Molecular formula	Reference
Sydowinol	$C_{16}H_{14}O_7$	Hamasaki, Sato & Hatsuda (1975b)
Sydowinin A	$C_{16}H_{12}O_6$	Hamasaki, Sato & Hatsuda (1975b)
Sydowinin B	$C_{16}H_{12}O_7$	Hamasaki, Sato & Hatsuda (1975b)
Sydonol	$C_{15}H_{24}O_3$	Kudo et al. (2009)
Hydroxysydonic acid	$C_{15}H_{22}O_5$	Hamasaki, Nagayama & Hatsuda (1978)
Sydonic acid	$C_{15}H_{22}O_4$	Hamasaki, Nagayama & Hatsuda (1978)
Sydowic acid	$C_{15}H_{20}O_4$	Hamasaki, Sato & Hatsuda (1975a)
Ergosta-7,22-dien-3β-ol	$C_{28}H_{46}O$	Ripperger & Budzikiewicz (1975); Wang et al. (2018)
Ergosterol	$C_{28}H_{44}O$	Venditti et al. (2017); Wang et al. (2018)
2-acetylaminobenzamide	$C_9H_{10}N_2O_2$	Baker & Almaula (1962); Wang et al. (2018)
Questin	$C_{16}H_{12}O_5$	Kimura et al. (1983); Wang et al. (2018)
β-Sitosterol	$C_{29}H_{50}O$	Ahmad, Ali & Alam (2010); Wang et al. (2018)
WIN 64821	$C_{40}H_{36}N_6O_4$	Barrow et al. (1993); Wang et al. (2018)
AGI-B4	$C_{16}H_{14}O_7$	Kim et al. (2002); Wang et al. (2018)
Pinselin	$C_{16}H_{12}O_6$	Healy et al. (2004); Wang et al. (2018)
Sydowin A	$C_{18}H_{14}Cl_2O_7$	Teuscher et al. (2006)
Sydowin B	$C_{18}H_{14}Cl_2O_6$	Teuscher et al. (2006)
Azaspirofurans A	$C_{22}H_{21}NO_7$	Ren et al. (2010)
Azaspirofurans B	$C_{22}H_{19}NO_7$	Ren et al. (2010)

(+)-dehydrosydonic acid	$C_{15}H_{21}O_4$	Lu et al. (2010); Wang et al. (2018)
Aspergillusene A	$C_{15}H_{22}O_2$	Trisuwan et al. (2011)
Aspergillusene B	$C_{15}H_{18}O_3$	Trisuwan et al. (2011)
(+)-(7S)-7-O-Methylsydonic acid	$C_{16}H_{24}O_4$	Trisuwan et al. (2011)
Aspergillusone A	$C_{16}H_{14}O_6$	Trisuwan et al. (2011)
Aspergillusone B	$C_{16}H_{16}O_8$	Trisuwan et al. (2011)
(Z)-5-(Hydroxymenthyl)-2-(6')-methylhept-2'-en-2'-yl)-phenol	$C_{15}H_{21}O_2$	Li et al. (2012); Wang et al. (2014)
Emodin	$C_{15}H_{10}O_5$	Hawas, El-Beih & El-Halawany (2012); Zhou et al. (2018)
Disydonol B	$C_{30}H_{46}O_5$	Sun et al. (2012); Wang et al. (2018)
(7S)-(+)-7-O-methylsydonol	$C_{16}H_{26}O_3$	Chung et al. (2013)
(7S,11S)-(+)-12-hydroxysydonic acid	$C_{15}H_{22}O_5$	Chung et al. (2013)
7-deoxy-7,14-didehydrosydonol	$C_{15}H_{22}O_3$	Chung et al. (2013)
Yicathin C	$C_{17}H_{14}O_6$	Sun et al. (2013); Wang et al. (2018)
13-O-acetylsydowinin B	$C_{18}H_{14}O_8$	Song et al. (2013)
Sydoxanthone A	$C_{18}H_{16}O_7$	Song et al. (2013)
Sydoxanthone B	$C_{18}H_{14}O_8$	Song et al. (2013)
Wentiquinone C	$C_{16}H_{13}O_7$	Li et al. (2014); Wang et al. (2018)
Aspergillusene C	$C_{15}H_{20}O_4$	Wang et al. (2014)
Anhydrowaraterpol	$C_{15}H_{20}O_3$	Henne et al. (1993); Wang et al. (2014)
cyclo-Waraterpol A	$C_{15}H_{22}O_3$	Henne et al. (1993); Wang et al. (2014)
(S)-(+)-dehydrosydonic acid	$C_{15}H_{20}O_4$	Lu et al. (2010); Wang et al. (2014)
(7S,11S)-(+)-12-acetoxysydonic acid	$C_{17}H_{24}O_6$	Lu et al. (2010); Wang et al. (2014)
Diorcinol	$C_{14}H_{14}O_3$	Fremlin et al. (2009); Wang et al. (2014)
Cordyol C	$C_{14}H_{14}O_4$	Bunyapaiboonsri et al. (2007); Wang et al. (2014)
Cyclo-(*L*-Trp-*L*-Phe)	$C_{20}H_{19}N_3O_2$	Kimura et al. (1996); Wang et al. (2014)
Ergosterol peroxide	$C_{28}H_{44}O_3$	Cantrell et al. (1999); Wang et al. (2018)
12-O-acetyl-AGI-B4	$C_{18}H_{16}O_8$	Elnaggar et al. (2016); Wang et al. (2018)
4-hydroxybenzaldehyde	$C_7H_6O_2$	Liu et al. (2018); Wang et al. (2018)
2-hydroxy-6-formyl-vertixanthone	$C_{16}H_{10}O_7$	Wang et al. (2018)
12-O-acetyl-sydowinin A	$C_{18}H_{14}O_7$	Wang et al. (2018)
13-O-acetylsydowinin B	$C_{18}H_{14}O_8$	Song et al. (2013)

2.3.4 Fungal strains from the 2009 CPR silks, and metabolites in CPR silk materials

Further investigation of the 2009 CPR silks supported the dominance of *A. sydowii*, but also detected a few minor species: 73.7% *A. sydowii*; 10.5% unknown species (based on HPLC analysis); 7.9% *Aspergillus* sp.; 5.3% *Penicillium* sp. (Table 2.3 and Figure 2.3). These strains were isolated using half strength malt extract agar (MEA) and tetracycline, and included *Cladosporium* sp. (from location 4) and *Penicillium* sp. (from location 3).

The isolated *Penicillium* strains produced both known metabolites, such as rugulosin and previously unencountered co-metabolite profiles, the latter suggesting a novel species. Additional unidentified species producing unknown metabolites were also encountered from locations 1 and 4. Further genetic and metabolic characterisation of those unknown species is in progress. Additionally, *Aspergillus* sp. producing sterigmatocystin was detected. HPLC analyses of isolated *A. sydowii* indicated varying metabolite profiles and some produced unknown metabolites on MEA; however, all isolates produced sydonic acid. Their metabolite profiles exhibited some degree of overlap with some, but not all, terrestrial *A. sydowii* strains.

Direct extraction of CPR silk materials provided a different perspective on the dominance of *A. sydowii*. The chromatographs of silks from 32.238–32.319°S, 152.884–152.842°E (location 2) and 32.701°S, 152.579°E (location 3) exclusively detected *Pseudomonas* phenazine metabolites, tubermycin (λ_{max} 248, 344 s, 370 nm) and oxychlorophine (λ_{max} 248, 344 s, 370 nm), while *A. sydowii* metabolites were present in only trace amounts (Appendix Figure A.2).

Table 2.3 Fungal strains isolated from the 2009 dust storm and secondary metabolites found by HPLC analysis of methanol extracts grown on malt extract agar (MEA). Numbers in brackets indicates the number of each species.

Location	Species	Metabolite(s)	% Isolates
1	*A. sydowii*	sydonic acid	5.3 (2)
		sydonic acid, sydonol	15.8 (6)
		sydonic acid, unknown metabolites[1]	7.9 (3)
	Penicilllium sp.	rugulosin	2.6 (1)
	Unknown 1	no detectable metabolites	5.3 (2)
2	*A. sydowii*	sydonic acid	7.9 (3)
		sydonic acid, sydowinin B	2.6 (1)
		sydonic acid, unknown metabolites[1]	10.5 (4)
	Aspergillus sp.	sterigmatocystin	2.6 (1)
3	*Penicillium* sp.	rugulosin	2.6 (1)
4	*A. sydowii*	sydonic acid	21.1 (8)
		sydonic acid, sydonol	2.6 (1)
	Aspergillus sp.	sterigmatocystin	5.3 (2)
	Cladosporium sp.	no detectable metabolites	2.6 (1)
	Unknown 2	unknown metabolite[2]	5.3 (2)
			Total 38 isolates

[1] λmax 230, 279, 288, 320 and 365 nm; [2] λmax 216 nm.

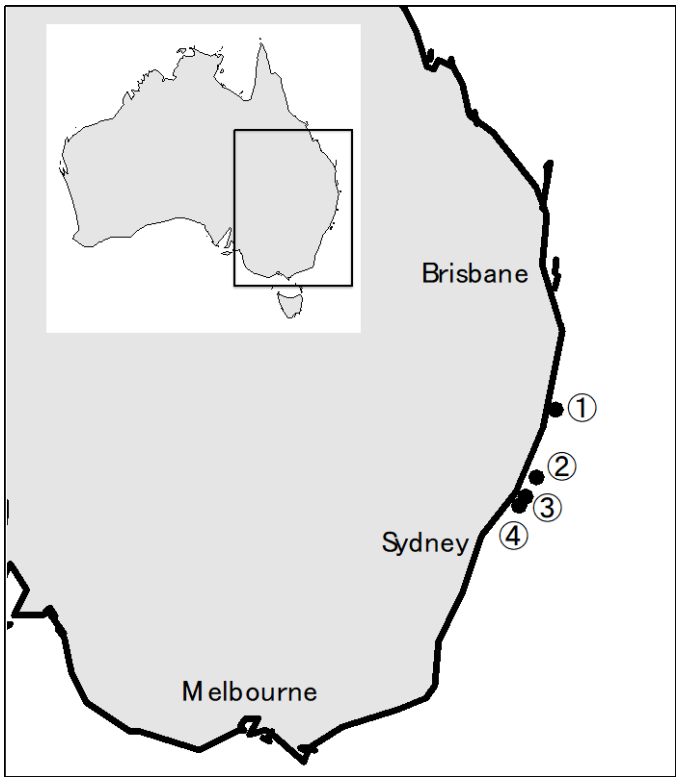


Figure 2.3 The distribution of fungal species in the 2009 dust storm. Latitude and longitude of each location (circled number from 1 to 4) are following: 30.617–30.703°S, 153.425–153.401°E (location 1), 32.238–32.319°S, 152.884–152.842°E (location 2), 32.701°S, 152.579°E (location 3), 32.917–32.989°S, 152.393–152.331°E (location 4).

2.3.5 Effect of Terrestrial and Marine *A. Sydowii* Crude Extracts on *Symbiodinium* Photo-Physiological State

Application of two concentrations (0.1 and 0.3 mg) of crude extracts of terrestrial (FRR5152) and 2009 dust storm originated (ASBS) *A. sydowii* had a minor effect on maximum quantum yield (F_v/F_m) of CS156 *Symbiodinium* (clade C) at day 2 ($F(4,10) = 27.2$, $p < 0.001$), exhibiting a significantly reduced F_v/F_m by 0.025 to 0.056, respectively, compared to the control (Figure 2.4). F_v/F_m is a measure of the maximum efficiency of photosystem II, used as an index of plant photosynthetic performance (Maxwell & Johnson 2000). At day 4, 0.1 mg FRR5152 and ASBS crude extracts exhibited significantly higher F_v/F_m of CS156 with increases of 0.051-0.054, whereas 0.3 mg FRR5152 and ASBS crude extracts continued to exhibit significantly lower F_v/F_m at day 4, with declines of 0.029-0.039 compared to

control values ($F(4,10) = 50.2$, $p < 0.001$). At days 6 and 8, there was no significant difference in F_v/F_m between treatment and control. However, there was a trend that F_v/F_m at 0.3 mg ASBS crude exhibited lowest values throughout the experiment period. There was no significant effect of crude extracts on the more resilient strain CS163 *Symbiodinium* (clade A1) F_v/F_m throughout the 8-day experimental period (results not shown).

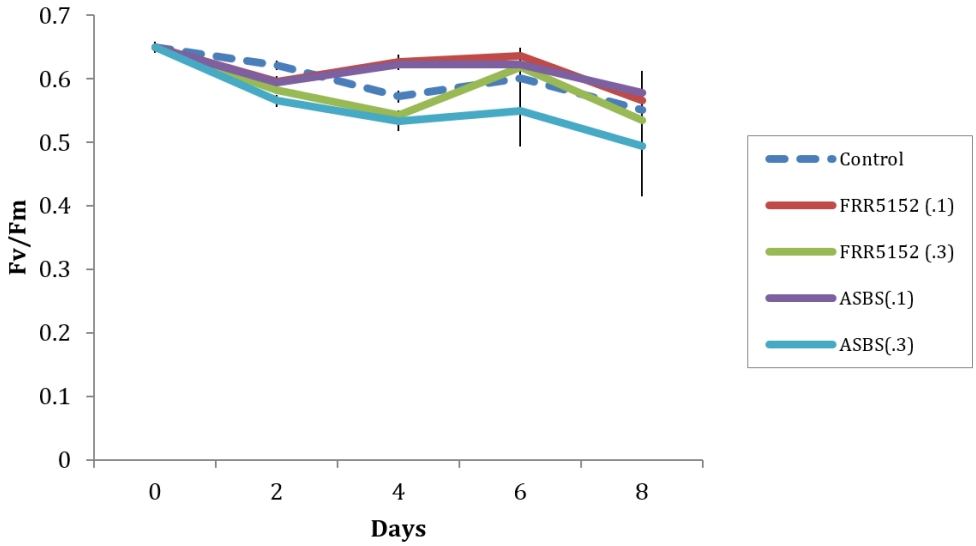


Figure 2.4 Effect of terrestrial (FRR5152) and 2009 dust originated *A. sydowii* (ASBS) crude extracts on CS156 *Symbiodinium* maximum fluorescent yield (F_v/F_m) during an 8-day period. Error bars represent sample standard deviation from triplicate measurements. (.1) and (.3) indicate 0.1 and 0.3 mg dosage respectively.

2.3.6 Effect of Known *A. sydowii* Metabolites on *Symbiodinium* Photo-Physiological State

Known *A. sydowii* metabolites had significant effects on F_v/F_m of CS156 (clade C) and CS73 (clade A), however caused no effect on CS163 (clade A1) (Figure 2.5). Treatment with 0.1 mg doses of typical metabolites significantly reduced F_v/F_m of CS156, whereas 0.01 mg dosage had no or minor effect on F_v/F_m on CS156 (from day 2–8, $F(8,18) = 65.58$ $p < 0.001$, at day 2 $F(8,18) = 254.6$, $p < 0.001$ at day 4, $F(8,18) = 1060.0$, $p < 0.001$ at day 6 and $F(8,18) = 253.9$, $p < 0.001$ at day 8). F_v/F_m of CS156 at 0.1 mg sydowinin A exhibited a gradual decline and lowest F_v/F_m of 0.370 ± 0.0256 at day 8. Application of 0.1 mg of sydowic acid also significantly decreased F_v/F_m to 0.455 ± 0.0061 at day 6. There was an increase at day 8

to 0.532 ± 0.0053. Application of 0.1 mg of sydowinin B caused a significant reduction in F_v/F_m to between 0.507 ± 0.0009 and 0.549 ± 0.0018, and application of sydowinol caused a reduction between 0.516 ± 0.0077 and 0.573 ± 0.016. Application of 0.01 mg of those metabolites caused a minor reduction in F_v/F_m of CS156 by 0.028 to 0.048 compared to the control. On the other hand, 0.1 mg of all typical *A. sydowii* metabolites except sydowinin B caused a significant effect on F_v/F_m of CS73, whereas 0.01 mg of metabolites had no significant effect from day 2 to day 8, $F(8,18) = 16.8$, $p < 0.001$, $F(8,18) = 45.5$, $p < 0.001$, $F(8,18) = 19.54$, $p < 0.001$ and $F(8,18) = 24.14$, $p < 0.001$). At day 2, only 0.1 mg sydowinin A caused a significant decline in F_v/F_m of CS73. After day 2 application of 0.1 mg of sydowinin A, sydowinol and sydowic acid significantly decreased F_v/F_m of CS73 to 0.517 ± 0.0031 (day 4), 0.458 ± 0.0157 (day 8) and 0.470 ± 0.0056 (day 8) respectively.

There was no significant effect from standard metabolites on CS163 *Symbiodinium* F_v/F_m throughout the eight-day experimental period (results not shown), except that at day 4. F_v/F_m of CS163 at 0.1 mg sydowinol caused significantly lower values than those at other treatments with a reduction of 0.041 compared to the control.

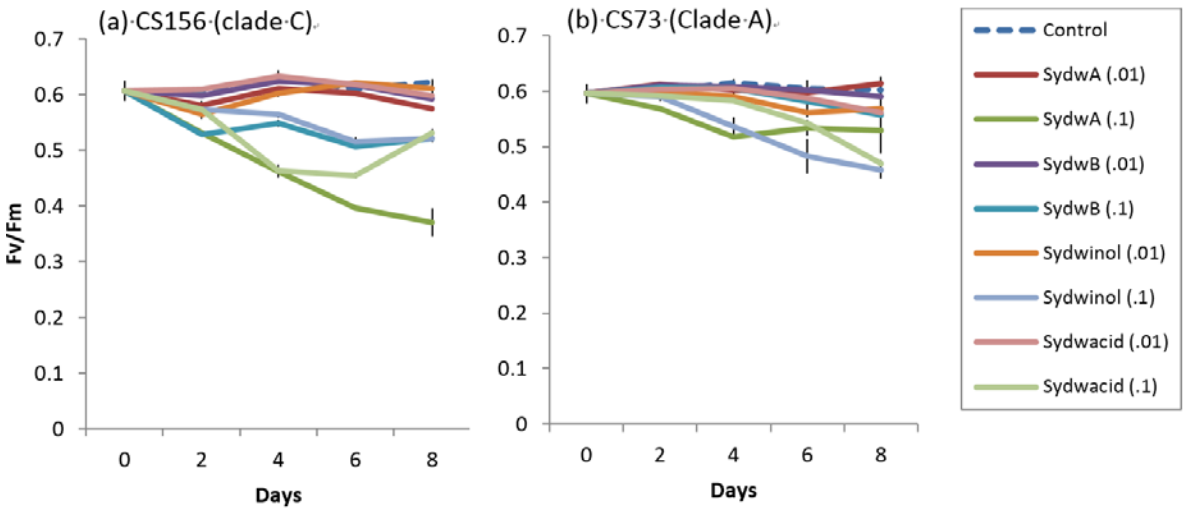


Figure 2.5 Effect of typical *A. sydowii* metabolites on CS156 (a) and CS73 (b) *Symbiodinium* maximum fluorescent yield (F_v/F_m) during an eight-day study period. Error bars are sample standard deviation from triplicate measurements. SydwA, SydwB, Sydwinol, and Sydwacid are sydowinin A, sydowinin B, sydowinol and sydowic acid, respectively. (0.01) and (0.1) indicate 0.01 and 0.1 mg dosage applied in this study.

2.4 Discussion

We show that the Australian marine *A. sydowii* strains share major metabolites and display comparable metabolic diversity to Australian terrestrial strains and to strains pathogenic to Caribbean coral. We also find secondary colonisation of the rafts by other *Aspergillus* species and fungal strains of *Cladosporium* and *Penicillium*. Our bioassays reveal that the dust-derived marine fungal extracts adversely affect photophysiological performance of the important coral reef dinoflagellate endosymbiont *Symbiodinium*. Different *Symbiodinium* clades exhibit varying sensitivities, mimicking sensitivity to coral bleaching phenomena.

2.4.1 Dust Generated Microbial Raft Ecosystem

Dust has been widely recognised as an important source of nutrient input into the marine environment and a vector for transporting pathogenic microorganisms (Garrison et al. 2003). The dust storm in 2009 covered a large area of the Australian coast, and created a distinctive marine raft micro-environment.

It is probable that the dust layer was sufficiently hydrophobic to remain un-wetted on the ocean surface for a considerable time, during which spores of many genera of saprophytic species germinated. The levels of fungal spores on dust should reflect typical levels and diversity of soil fungi and have a density of 10^4 to 10^6 spores per gram (colony forming units) with considerable species diversity >>100 (Tedersoo et al. 2014). Surface culture of fungi is itself not new; forming a raft on liquid media dates to the discovery of penicillin and is a fundamental technique in fungal cultivation. Most, but not all, saprophytic fungi form rafts on stationary liquid culture and these surfaces are profoundly water repellent. In cultivation studies using cellophane rafts on agar media, it was noted that many fungi grown on concentrated media (hypertonic, hyperosmotic, high nutrient) simply did not produce secondary metabolites, however, interceding with a thin cellophane membrane led to massive increases in the abundance and diversity of secondary metabolites (Fremlin et al. 2009).

Based on viable spores recovered from trawling silks of the raft masses, *A. sydowii* was the dominant species, with additional species of *Aspergillus*, *Penicillium* and *Cladosporium* accumulated as secondary colonisers. These fungi are well known as terrestrial species, but are also occasionally isolated from marine environments (Jones 2011; Morrison-Gardiner 2002). Two previous studies have reported the commonly observed taxa, *Aspergillus* spp., *Cladosporium* spp. and *Penicillium* spp., in the Australian marine environment from sources such as sediment, algae and invertebrates (Andreakis et al. 2015; Morrison-Gardiner 2002). Furthermore, Toledo-Hernández *et al.* isolated *Penicillium* spp. from both healthy and unhealthy coral *Gorgonia ventalina*, which is one of the most abundant species in Puerto Rico (Toledo-Hernández et al. 2008). These authors similarly reported that both *Penicillium* and *Aspergillus* were the most abundant fungal species in the corals sampled. Fungal communities are a normal feature of healthy reefs, but the occurrence of massive dust-induced fungal rafts may lead to an infection event.

2.4.2 Secondary Metabolites Associated with Pathogenic and Non-Pathogenic Strains of *A. sydowii*

While the HPLC traces of terrestrial and marine *A. sydowii* show some differences in their co-metabolite profiles, the major known metabolites, constituting >50% of the total co-

metabolite AUC, are shared. Importantly, the metabolites reflect our general understanding of *A. sydowii* chemistry. Most obvious are the highly characteristic UV spectra of the sydonic acids, sydowinin and sydonols. These metabolites are the chemical framework of every strain investigated in this study and while the strains all share this profile, they are not identical. *A. sydowii* is capable of producing large numbers of secondary metabolites, but most are detectable in only trace levels. Currently, >30 metabolites from *A. sydowii* strains have been published in the literature, however the role and bioactivity of these are largely undescribed. The Australian marine strain displayed a more streamlined metabolite distribution, which suggests intensive strain selection on marine adaptation. A similar trend has been reported by Malmstrøm *et al.* who detected similar co-metabolite profiles between pathogenic and non-pathogenic strains (Malmstrøm et al. 2001).

2.4.3 Effect of Fungal Crude Extracts and *A. sydowii* Typical Metabolites on *Symbiodinium* Photophysiology

F_v/F_m is an indicator of the efficiency of photosystem II charge separation, which determines photophysiological performance of algal species (Maxwell & Johnson 2000). A decrease in F_v/F_m implies stress to photochemical efficiency (Burns, Gregg & Takabayashi 2013; Maxwell & Johnson 2000). Declines in *Symbiodinium* F_v/F_m might be significant to the coral host, as endosymbiont photosynthesis supports coral metabolism and provides a source for carbon (Dubinsky & Berman-Frank 2001). Our result is consistent with a study that compared *in situ* F_v/F_m of *Symbiodinium* from growth anomaly diseased and healthy coral individuals with a reduction of 1.5 in F_v/F_m (Burns, Gregg & Takabayashi 2013). Similarly, other coral disease studies revealed that *Vibrio* bacteria, a pathogen of yellow band disease, caused significantly decreased *Symbiodinium* chlorophyll *a* and c_2, and increased occurrence of lysed cells of *Symbiodinium* (Ben-Haim, Zicherman-Keren & Rosenberg 2003; Cervino et al. 2004). In addition, aspergillosis-infected coral tissue harboured fewer *Symbiodinium* cells compared to healthy coral tissues (Kirk, Ward & Coffroth 2005). These findings add support to our conclusion that *Symbiodinium* coral endosymbionts are negatively impacted by typical *A. sydowii* fungal metabolites.

The bioassay results showed that typical *A. sydowii* metabolites, including sydowinin A, sydowinin B, sydowinol and sydowic acid, significantly reduced F_v/F_m of *Symbiodinium* dinoflagellates. Sydowinin A (impacting on CS156 clade C) and sydowinol and sydowic acid (impacting on C73 clade A) were the most active. Previously, *A. sydowii* from the marine sponge *Spongia obscura* has infected the *Gorgonia ventalina,* and this strain produced typical metabolites such as sydonic acid and sydowic acids, 6-*O*-methylsydonic acid, 6-*O*-methyl-13-hydroxysydonic acid and diorcinol (Ein-Gil et al. 2009). This suggests that pathogenicity could be determined by secondary metabolites. The crude extract bioassays exhibited less clear effects on *Symbiodinium* F_v/F_m, which might be due to the presence of different interacting compounds. However, there was a trend that marine-originated *A. sydowii (*ASBS) had more impact on *Symbiodinium* than the terrestrial strain.

Symbiodinium is known to be extremely diverse genetically; eight distinct genetic clades (A–H) have been characterised (Pochon et al. 2006). This genetic variation plays key functional roles in behavioural, biochemical and physiological variation of coral hosts (Stat, Morris & Gates 2008; Yamashita et al. 2011). Our study revealed a difference in responses between *Symbiodinium* clades. Strains CS156 clade C and CS73 clade A exhibited high and moderate sensitivities, respectively, whereas CS163 clade A1 showed low sensitivity. Similarly, *Symbiodinium* stress-tolerant clade A and E dominated the bleached tissues of yellow blotch diseased corals whereas narrowly adapted specialists clade B and C dominated healthy tissues of corals (Toller, Rowan & Knowlton 2001). In contrast, growth anomaly coral disease and sea fan aspergillosis harboured consistent *Symbiodinium* clade types regardless of disease infection status (Burns, Gregg & Takabayashi 2013; Kirk, Ward & Coffroth 2005). Caribbean octocorals harbour mostly *Symbiodinium* clade B and rarely clade C, whereas those from the Australian Great Barrier Reef (GBR) harbour predominantly clades C and D with clades A, B, G much less abundant (Van Oppen et al. 2005). Clade B might be as sensitive as clade C towards typical *A. sydowii* metabolites since they are closely related genetically and have been defined as narrowly adapted specialists causing yellow blotch disease. Furthermore, clade C was the most sensitive *Symbiodinium* clade in this study. The fact that the Great Barrier Reef has not experienced significant fungal coral disease events to

date (Willis 2015) suggest that the high diversity of octocoral communities reflects a less impacted coral reef community compared to the Caribbean.

2.4.4 Conclusion

The marine strain of *A. sydowii* isolated after a 2009 dust storm on the eastern Australian seaboard represents a streamlined secondary metabolite profile that shares the same major co-metabolites of its terrestrial parent. These major co-metabolites significantly reduced *Symbiodinium* photo-physiological state. This effect may constitute a key mechanism for the effect of sea fan aspergillosis on the coral host. However, there is also evidence that the marine *A. sydowii* is more toxic to *Symbiodinium* than the terrestrial strain, suggesting important roles for other co-metabolites in the *A. sydowii* repertoire. Furthermore, there was a clade-dependent degree of sensitivity of *Symbiodinium* to *A. sydowii* metabolites and crude extract which mimics the sensitivity of corals and their symbionts to coral bleaching. Work in progress aims to characterise novel strain-specific *A. sydowii* metabolites and examine their role in driving differing virulence by terrestrial and marine, Australian and Caribbean fungal strains.

Chapter 3. Assessment of possible impact of mycotoxin on marine animals using fish gill cells as a model

Abstract

Fungal pathogens are increasingly recognised as an emerging threat to marine ecosystems. Our current knowledge of their impacts is limited compared to terrestrial ecosystems. The current chapter assessed the impact of dust storm and shellfish associated mycotoxins on the fish gill cell line (RTgill-W1), and their possible synergisms with algal toxins. Based on calculated IC_{50} (inhibitory concentration 50%) values, mycotoxins exhibited increasing cytotoxicity in the order of sydowinin A << sydowic acid < patulin < sydowinol < alamethicin <<gliotoxin. The mycotoxins sydowinin B and sterigmatocystin, and the algal toxins saxitoxin and domoic acid exhibited no cytotoxicity. Furthermore, the previously reported domoic acid and peptaibol synergism, using a Diptera bioassay (Ruiz et al. 2009), was not observed in the current bioassay using the fish gill cell line. The current fish gill cell model studies suggest the potential of fish mortality from marine mycotoxins. This could occur under conditions where aqueous phase mycotoxins come into close contact with sensitive gill cells.

3.1 Introduction

Fungi are often isolated from the marine habitat such as mangroves (Kohlmeyer 1969), sediments (Morrison-Gardiner 2002; Zhang et al. 2015), and marine organisms such as corals (Barrero-Canosa, Dueñas & Sánchez 2013), sponges (Ein-Gil et al. 2009) and algae (Morrison-Gardiner 2002). Despite their frequent occurrence in marine habitats, our understanding of fungi in marine environments is scarce. Currently 530 isolates have been derived from marine environments (Rateb & Ebel 2011) which implies that only ~0.6 % of total studied fungi have been isolated from marine environments (Richards et al. 2012). Furthermore, our current understanding of fungal ecology and evolutionary complexity is largely based on the studies of isolates from terrestrial environments (Richards et al. 2012).

Similar to fungi in terrestrial environments, those in marine environments are known to play diverse ecological roles including decomposers, parasites and pathogens (Hyde et al. 1998). Fungal infections often cause fatal damage to the organisms, and they are often difficult to treat (Noga 1990). For example, sea fan coral aspergillosis is an epizootic disease of sea fan corals, *Gorgonia* spp. associated with the fungal pathogen *Aspergillus sydowii* (Kim & Harvell 2004). This fungal disease caused decline in coral coverage by approximately 50 % in Mexico (Kim & Harvell 2004), and caused 20 to 90 % of sea fan mortalities in Florida Keys (Bruno et al. 2011). Other than sea fan corals, fungi have been identified as pathogens to other marine organisms such as coralline algae (Littler & Littler 1998), coral (Smith et al. 1996), sea turtles (Sarmiento-Ramírez et al. 2010), fish and prawns (Hatai 2012). The majority of research on fungal disease in marine environment has extensively focused on economically valuable aquaculture (Noga 1990) or their etiology (Correa et al. 2009), however less on their ecological roles or nature of interaction with hosts (Amend et al. 2019). A few recent studies explored broader ecological aspects of marine fungi such as species composition in response to the environmental stress (Amend, Barshis & Oliver 2012), species diversity in plankton, wetland sediment, intertidal sand and sediment core (Picard 2017). Nevertheless, their roles in the marine environment and detailed interaction with hosts have remained unclear.

Marine fungal infections are often described as opportunistic (Mohamed et al. 2017; Mydlarz et al. 2008; Sarmiento-Ramírez et al. 2014) which indicates that fungi mainly infect immunocompromised hosts (Radentz 1989). Human activity and climate change are major factors that affect host immunity in marine environments. The current warming trend could compromise environmental health and productivity while human disturbance often induces pollutant and habitat degradation (Harvell et al. 1999). Therefore, in the marine environment, fungal disease is predicted to increase due to climate change and human disturbances. Furthermore, aquaculture feeds are new vectors of introducing mycotoxins to the marine environment. Due to the recent increased use of plant derived proteins for aquaculture feeds (Francis, Makkar & Becker 2001), they are often contaminated (>51 %) with fungi and their mycotoxins including aflatoxins (AF), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB) and ochratoxins (OTA) (Barbosa et al. 2013; Gonçalves et al. 2017; Gonçalves, Naehrer & Santos 2016; Pietsch et al. 2013). Typical effects of these mycotoxins on fish include feed efficiency, damage to organs, neurotoxicity and mortality (Anater et al. 2016). For example, DON has been reported to decrease growth performance such as feed intake, weight gain, growth rate and feed efficiency, cause subcapsular hemorrahage of the liver, and affects body protein content on rainbow trout (*Oncorhynchus mykiss*) (Hooft et al. 2011).

RTgill-W1 gill cell line has been suggested as a suitable model of assessing aquatic toxicology to *in vivo* whole fish (Lee et al. 2009). First, this RTgill-W1 cell line was originated from rainbow trout (*O. mykiss*) (Bols et al. 1994). Rainbow trout has both a marine and freshwater life cycle, hence the RTgill-W1 cell line can be a suitable model for assessing both freshwater and marine toxicology. Secondly, the gills of aquatic animals serve multiple important processes such as gas exchange, osmoregulation, pH regulation and nitrogen balance (Evans 2005). These functions are critical for their survival, and serve as the primary target sites for water-born contaminants to fish (Tanneberger et al. 2013). Hence, damage to the gill could reflect their death (Lee et al. 2009). This is further supported by studies by Tanneberger *et al.* (2012) and Mooney *et al.* (2011). Tanneberger *et al.* (2012) demonstrated an overall good agreement between *in vivo* fathead minnow and *in vitro* RTgill-W1 toxicity. Furthermore, Mooney *et al.* (2011) reported higher sensitivity of RTgill-W1 to fatty acid

produced by phytoplankton compared to sheepshead minnow fish larvae. Lastly, fish gill cell line models are also advantageous over *in vivo* models in terms of easy maintenance, experimental flexibility and high reproducibility (Lee et al. 2008). This advantages could represent their wide applicability in toxicological studies. RT-gillW1 have been successfully used for the assessment of ichthyotoxity of harmful algae (Dorantes-Aranda et al. 2011; Mardones et al. 2015; Seger et al. 2015), emerging mycotoxins in aquaculture (Mayer et al. 2017), heavy metal (Bopp, Abicht & Knauer 2008), and infectious salmon anemia virus (Falk et al. 1997).

There are increasing risks of impacts from fungi in marine environments due to climate change, human disturbances and aquaculture feeds. The 2009 dust storm associated *A. sydowii* "bloom" and other co-occurring fungi raised the alarm of possible risks. Moreover, the knowledge gap in the impacts of fungi on marine organisms would be crucial under such scenarios. The present study aimed to assess the impact of dust storm associated *A. sydowii* metabolites, dust storm and farmed tiger shrimp associated mycotoxin sterigmatocystin (See Chapter 3 for details; Fernand et al. 2017) and shellfish associated mycotoxins such as gliotoxin (Grovel, Pouchus & Verbist 2003), alamethicin (Poirier, Montagu, et al. 2007) and patulin (Vansteelandt et al. 2012) on the fish gill RTgill-W1 cell as a model. Their basic characteristics such as mode of actions, toxicities and chemical structures are summarised in Table 3.1 and Figure 3.1. Previously reported mycotoxin and phycotoxin interactions using a Diptera larvae bioassay (Ruiz et al. 2010) were also assessed against the RTgill-W1 cells.

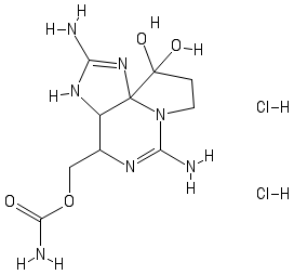
Table 3.1 Algal toxin, dust storm and shellfish associated mycotoxins tested in this study.

Toxin	LD_{50}[1] in mouse (mg/kg)	IC_{50}[2] cytotoxicity (mammalian cell)	Mode of action
Algal toxin			
Saxitoxin	0.01 (Wiberg & Stephenson 1960)	1.01 nM - no effect on Neuro-2a (Cañete & Diogène 2008; Melegari et al. 2015)	Saxitoin binds to voltage-gated sodium channels on nerves and muscles preventing nerve impulse (Wood, Longley-Wood & Reenstra 2016).
Domoic acid	3.6 (Grimmelt et al. 1990)	~8 µM on primary cultures of cerebellar granule cells (Antonello Novelli et al. 1992).	Domoic acid activates glutamate receptors in the postsynaptic membrane, triggering Ca^{2+} influx into neurons (Pulido 2008)
Mycotoxin			

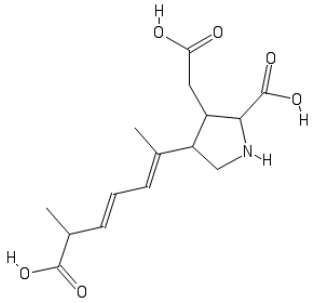
Patulin	7.6 (Hayes et al. 1979)	2.9 µM on CHO-K1 cells (Zouaoui et al. 2016)	Patulin causes oxidation of sulfhydryl group in cell membranes. This decreases intracellular potassium and causes unbalance of ion concentrations across the cell membrane (Riley & Showker 1991)
Gliotoxin	32 (Larin et al. 1965)	0.3 µM on A549 cell (Kreja & Seidel 2002)	Gliotoxin selectively binds to cytoplasmic membrane thiol groups. This alters membrane protein orientation could result in increased membrane permeability (Jones & Hancock 1988)
Sterigmatocystin	800 (Davis 1981)	25 µM on CHO-K1 cells. (Zouaoui et al. 2016)	Sterigmatocystin uncouples the oxidative phosphorylation process (Kawai et al. 1984). It also inhibits acyl-CoA:cholesterol acyltransferase (ACAT) which maintains cellular cholesterol levels (Chang et al. 2009).
Alamethicin (Peptaibol)	80 (Meyer & Reusser 1967)	16 µM on human erythrocytes (Brückner, Graf & Bokel 1984)	Alamethicin forms pores in membrane (Mueller & Rudin 1968), increasing membrane permeability. It also forms voltage-gated ion channels in membranes under certain conditions (Tamm & Tatulian 1997).

sydowinin A, sydowinin B, sydowic acid and sydowinol	N/A	N/A	No reported mechanism of action

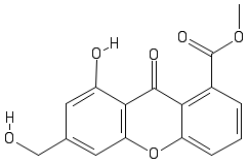
[1]LD_{50} indicate lethal dose 50%. [2]IC_{50} indicate inhibitory concentration 50%.



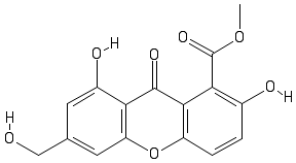
Saxitoxin



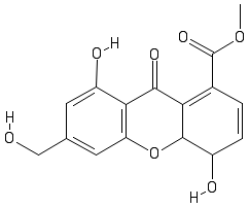
Domoic acid



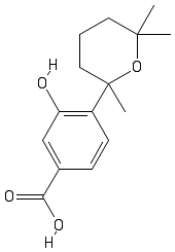
Sydowinin A



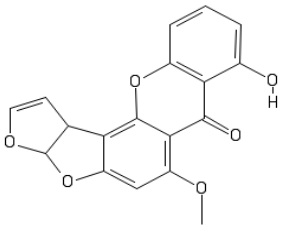
Sydowinin B



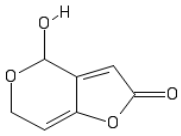
Sydowinol



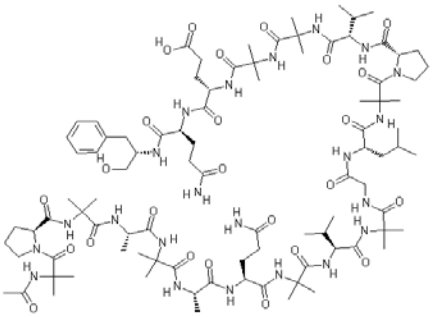
Sydowic acid



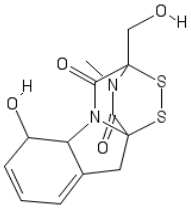
Sterigmatocystin



Patulin



Alamethicin



Gliotoxin

Figure 3.1 Chemical structures of typical algal toxins (saxitoxin, domoic acid), dust storm related mycotoxins (major *A. sydowii* metabolites) and dust storm and farmed fish associated mycotoxins (sterigmatocystin) and shellfish related mycotoxins (patulin, alamethicin, gliotoxin).

3.2 Materials and methods

3.2.1 Fish gill cell culture

RTgill-W1, the rainbow trout *Oncorhynchus mykiss* gill cell line, was acquired from the American Type Culture Collection. The cells were maintained at 20ºC in the dark with Leibovitz's medium (L1518, Sigma-Aldrich) supplemented with 10% foetal bovine serum (v/v) and an antibiotic-antimycotic solution (A5955, Sigma-Aldrich) with penicillin (10,000 units/ml), streptomycin (10 mg/ml) and amphotericin B (25mg/ml). Gill cells were routinely sub-cultured at a ratio of 1:3 and medium change occurred every 3 d.

3.2.2 Mycotoxin and algal toxin

Four typical *A. sydowii* metabolite standards sydowinol, sydowinin A, sydowinin B, sydowic acid were provided by Professor Hiromitsu Nakajima, Tottori University, Japan. These compounds were isolated from *A. sydowii* IFO 4284 and IFO 7531 strains. Details of UV, IR and NMR spectra, chemical structures and molecular weights were described in Hamasaki, Sato & Hatsuda (1975a, 1975b). These crystallised metabolites were dissolved in acetone. Among the other mycotoxins tested, gliotoxin (G9893, Sigma-Aldrich) was dissolved in ethanol, alamethicin (A4665, Sigma-Aldrich) was dissolved in DMSO, and sterigmatocystin (S3255, Sigma-Aldrich) and patulin (P1639, Sigma-Aldrich) were dissolved in acetonitrile. All the mycotoxins purchased from Sigma-Aldrich were above 98% purity level. Phycotoxin standards, saxitoxin calibration solution (CRM-STX-f) was purchased from National Research Council Canada, and domoic acid (D6152, Sigma) was purchased from Sigma, and dissolved in sterile Milli-Q water. Saxitoxin used in this study was calibration standard, and purity level of domoic acid was above 90%.

3.2.3 Cytotoxicity bioassays

Exposure experiments followed the protocol developed by Dorantes-Aranda et al. (2011). Briefly, cells were seeded at the concentration of 2×10^5 cells/ml in a 96 well plate and incubated for 48 h prior to the toxin exposure. Dissolved toxins were added to a modified L-15 medium (L-15/ex) (Schirmer et al. 1997). Gill cells were washed with phosphate buffer saline (PBS) and exposed to L-15/ex containing toxins for 2 h. Tested concentration ranges were 3.83×10^{-6} to 642 µM for individual toxicity and 5.1×10^{-4} to 25.5 µM for combined toxicity. The control included solvents only, and concentrations used in the assay were

preliminary tested to have no significant effect on the cell viability compared those treated basal L-15/ex without solvents (data not shown). The toxin-containing medium was discarded, and gill cells were rinsed once with PBS to remove all the remaining toxins and media. 5% resazurin solution (v/v) in L-15/ex were added to each wells after PBS washing. Following additional 2 h incubation in the dark, fluorescence was measured by a microplate reader (FLUOstar OPTIMA, BMG Labtech, 413–3350), using excitation and emission filters of 540 and 590 nm. A 2 h exposure time was chosen to be comparable with other toxicities of a wide range of algal toxins determined by Dorantes-Aranda et al. (2015). A 2 h exposure time has previously been proven to be sensitive enough to demonstrate the effects of toxic algal live cultures of *Chattonella marina*, *Karlodinium veneficum* and *Prymnesium parvum*o on RTgill-W1 cells (Dorantes-Aranda et al. 2011; Seger et al. 2015). Viability of gill cells was calculated as percentage of mean fluorescence relative to the control cells (% of control).

3.2.4 Statistical analysis of cytotoxicity of mycotoxin and algal toxin

Dose response curves were fitted with the four-parameter logistic model (4PL), and 95% asymptotic confidence intervals were calculated using GraphPad Prism 7. The half-maximal inhibitory concentration (IC_{50}) indicating the concentration that caused a half-maximal viability was calculated for each toxin. One-way analysis of variance (ANOVA) was used to examine the statistical difference between control and treatments followed by Tukey's honestly significant difference (HSD) post hoc tests. Box–Cox transformation was applied to determine appropriate transformation to improve normality and homogeneity of variance. The extra sum-of-squares F test was performed to evaluate the statistical difference in IC_{50} values between treatments. A significance level of 0.05 was applied in this study.

3.3 Results

3.3.1 Individual cytotoxicity of algal toxin and mycotoxin

Cytotoxicity of individual mycotoxin and phycotoxins was tested against the fish gill cell line RTgill-W1 using resazurin cell viability reagent. Tested mycotoxins except

sydowinin B and sterigmatocystin displayed a dose-dependent effect with an IC_{50} of 0.098-103.7 µM (Figures 3.2A, B). The tested concentration of sydowinin B and sterigmatocystin had no significant effect on the viability (sydowinin B: $F(5,18) = 2.089$, $p = 0.114$, sterigmatocystin: $F(4,15) = 1.727$, $p = 0.196$). Similarly, tested concentration range of the algal toxins, saxitoxin and domoic acid had no significant effect on the viability of fish gill cells (saxitoxin: $F(7,24) = 1.217$, $p = 0.332$, domoic acid: $F(10,33) = 2.171$, $p = 0.0461$) (Figures 3.2C and 3.3). The HSD result indicated no significant difference between domoic acid treatments and control. Based on the calculated IC_{50} values, tested mycotoxins and algal toxins could be ranked in increasing order of sydowinin A << sydowic acid < patulin < sydowinol < alamethicin <<gliotoxin in gill RTgill-W1 cells. Toxicities of the tested algal toxins and mycotoxins in the present study are summarised in Table 3.2.

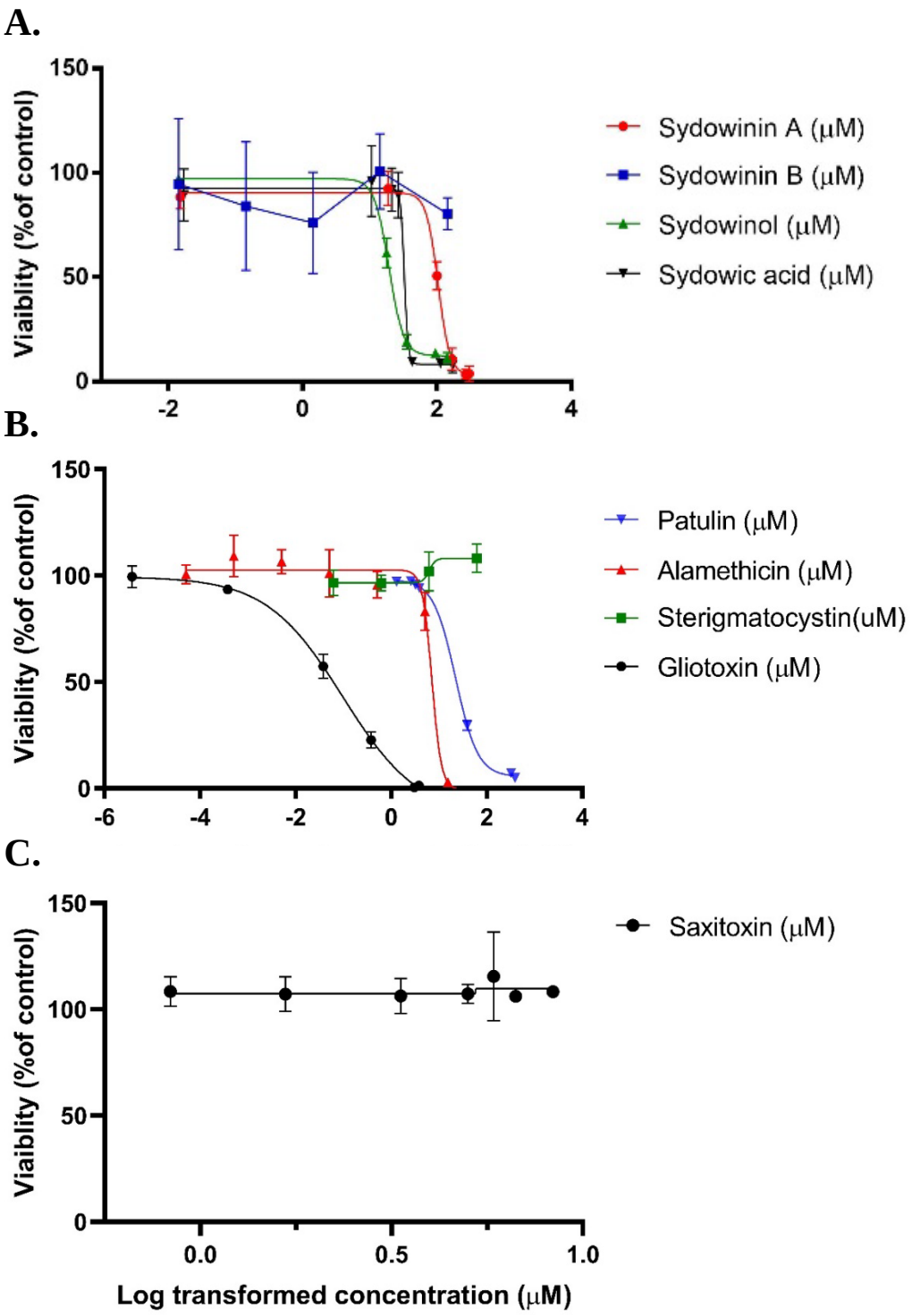


Figure 3.2 (A) Major *A. sydowii* metabolites, sydowinin A, sydowinin B, sydowinol and sydowic acid (B) mycotoxins, patulin, alamethicin, sterigmatocystin and gliotoxin (C) algal toxin saxitoxin dose-responses for cytotoxicity against fish gill RTgill-W1 cells. Toxin concentration was log transformed. Data are mean ± SD of four biological replicates.

Table 3.2 Summary of cytotoxicity of typical *A. sydowii* metabolites, other mycotoxins, and algal toxin,saxitoxin and domoic acid on fish gill RTgill-W1 cells after 2h exposure. IC_{50} values and 95% Confidence Intervals (CI) were calculated from four biological replicates using 4PL model.

Toxin	IC_{50} (μM)	95% CI
Typical A. sydowii *metabolites*		
Sydowinin A	103.7	99.51-108.00
Sydowinin B	NE (142.3)[1]	-
Sydowinol	19.19	18.45-19.97
Sydowic acid	32.67	27.08-39.42
Dust storm/shellfish mycotoxins		
Sterigmatocystin	NE (61.7)[1]	-
Patulin	22.31	19.66-25.32
Alamethicin	7.13	5.90-8.60
Gliotoxin	0.098	0.062-0.153
Algal toxins		
Saxitoxin	NE (8.35)[1]	-
Domoic acid	NE (642)[1]	-

[1]NE indicates toxins had no significant effect within the tested concentration range. Numbers in brackets indicate the maximum applicable concentration tested.

3.3.2 Alamethicin toxicity was not enhanced by domoic acid

Serial concentrations of alamethicin ranging from 5.1×10^{-4} to 25.5 µM and a constant concentration of domoic acid (6.4 µM) were together to test whether the mixture have the combined effects on gill cell viability (Figure 3.3). The differences between calculated IC_{50} values for domoic acid alone and the values for combined domoic acid and alamethicin were not significant (F(DFn, DFd) = $F(1, 60) = 0.0207$, p=0.886). The data indicated that there is no alterations in the inherent toxicity of alamethicin in combination with domoic acid on fish gill.

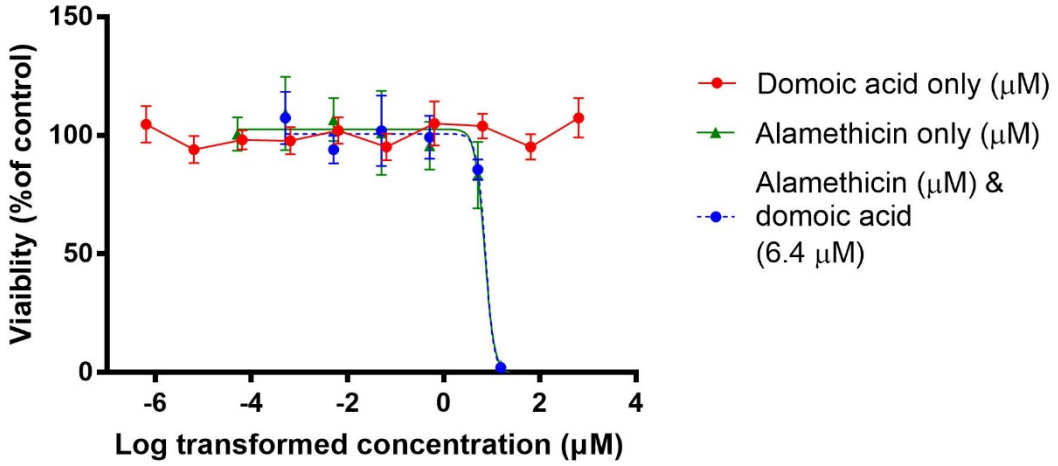


Figure 3.3 Alamethicin, domoic acid and combined alamethicin and constant dose of domoic acid (6.4 µM) dose responses for cytotoxicity against fish gill RTgill-W1. Toxin concentration was log transformed. Data are mean ± SD of four biological replicates.

3.4 Discussion

In the current study, dust storm and shellfish associated mycotoxins exhibited cytotoxicity to fish gill RTgill-W1 cells. The rank order, from highest to lowest, of individual mycotoxin cytotoxicity was gliotoxin, alamethicin, sydowinol, patulin, sydowic acid and sydowinin A. Tested concentration ranges of sterigmatocystin, sydowinin B, saxitoxin and domoic acid had no significant impact on viability of RTgill-W1 cells. Furthermore, potentiation of domoic acid and alamethicin was not observed in this study.

3.4.1 Marine mycotoxin cytotoxicity on fish gill RTgill-W1

Due to the recent increase in plant protein use in fish feeds, impacts of frequently occurring mycotoxins in crop (e.g. AF, ZEA, DON, FB) have been reported and assessed in laboratory settings (Matejova et al. 2017). However, impacts of emerging mycotoxins tested in the current chapter on marine organisms have been assessed to very limited extent. The present results exhibited an IC_{50} ranging from 0.098 to 103.7 µM. These are in agreement with previously reported cytotoxicity of other mycotoxins on RTgill-W1 cells. Gliotoxin, DON and ZEN exhibited IC_{50} of 0.28, ~40 and >62.8 µM, respectively (Dayeh et al. 2005; Mayer et al. 2017; Pietsch et al. 2014). Similarly, AFB_1, DON, and ZEN displayed IC_{50} of 11.1, 15.6 and 170.24 µM, respectively on fish caudal BF-2 cell line after 48 h exposure time (Zhou et al. 2017). The cytotoxicity rank order of shellfish and dust storm associated mycotoxins in the present study also corroborated mouse intraperitoneal LD_{50} values and other mammalian IC_{50} values except that fish gill cells were more sensitive to gliotoxin than the mouse bioassay. Although we lack toxicity information of the tested mycotoxins on whole fish, the present experimental data suggest the potential of fish mortality by mycotoxins especially if they reach sufficiently high aqueous concentrations. We currently lack knowledge of mycotoxin concentration in natural marine environments, except for a single report of 5ng/g in sediment samples (Poirier, Amiard, et al. 2007).

The major *A. sydowii* metabolite, sydowinol, sydowic acid and sydowinin A exhibited cytotoxicity against the fish gill RTgill-W1. Similarly, some *A. sydowii* metabolites displayed cytotoxicity against several human cell lines such as A549 (lung cancer cell), HeLa (cervical carcinoma cell), MDA-MB-435 (melanoma cell), HT-29 (colon cancer cell), KB (cervix carcinoma cell), HepG2 (liver cancer cell) and HCT116 (colon cancer cell). Some of these metabolites also exhibited toxicity towards brine shrimp larvae (Liu et al. 2017), and damage the photosynthetic property of *Symbiodinium* dinoflagellates (Chapter 2). Other reported biological properties of some *A. sydowii* metabolites include immunosuppressive activities (Liu et al. 2016; Song et al. 2013), antimicrobial and antiviral activities (Wang et al. 2014), inhibitory activity against *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) (Liu et al. 2013) and antioxidant activity (Trisuwan et al. 2011). Inactivity of sydowinin B on gill cells is in accordance with the previous chapter where sydowinin B was the only

metabolite that exhibited less effects on the dinoflagellate *Symbiodinium* photo-physiological state (F_v/F_m) (Chapter 2). Sydowinin B also exhibited weaker immunosuppressive activity compared to sydowinin A (Liu et al. 2016). Sydowinin A and B share similar chemical structures, however they differ in the presence of a hydroxyl group which might contribute to the observed difference in cytotoxicity (current study), effects on photophysiological states (Chapter 2) and immunosuppressive activity (Liu et al. 2016). We conclude that *A. sydowii* metabolites display a wide range of biological activities and cytotoxicity on various cell lines, however we lack knowledge on detailed mechanisms on how these metabolites impact cells, and the environmental conditions that trigger their production. This is concerning as *A. sydowii* can be readily isolated from marine environments including sandy beach soil (Latiffah Zakaria et al. 2011), deep seas (Wang et al. 2019), and animals such as corals (Soler-Hurtado et al. 2016) and sponges (Ein-Gil et al. 2009), and have previously caused significant mass mortality of sea fan corals.

3.4.2 Effects of algal toxins and their specific modes of action

The tested algal toxins, saxitoxin and domoic acid exhibited no effect on gill cell viability. This insignificant impact of saxitoxin on RTgill-W1 is in accordance with studies by Mardones et al. (2015, 2018) that showed minor effect on the viability and ion fluxes of RTgill-W1 cells. Both saxitoxin and domoic acid are categorised as neurotoxins which specifically target ion channels and receptors in brain and muscle. Saxitoxin binds voltage-gated sodium channel in nerves and muscle which eventually leads to respiratory paralysis and death in the worst case scenario (reviewed in Evans, 1972). Domoic acid selectively activates glutamate receptors (GluRs) in brain synapses and triggers excessive release of endogenous glutamate which results in cell and tissue injury (reviewed in Pulido, 2008). The neurotoxic symptoms and lethal toxicities of saxitoxin and domoic acid to fish were also observed under experimental conditions (Lefebvre, Trainer & Scholz 2004; Nogueira et al. 2010). While both saxitoxin and domoic acid have specific neurotoxic effects which could lead to fish death, they cannot significantly damage gill cells. Similarly, other neurotoxins such as permethrin (190-fold), lindane (63-fold) and caffeine (18-fold) also have exhibited less effects on RTgill-W1 compared to overall acute fish toxicity (Tanneberger et al. 2013)

while other toxicants showed a good agreement between *in vivo* and *in vitro* test results. Tanneberger et al. (2012) argued that lack of neurotransmitter target sites on gill cells, as opposed to sodium or chloride channels in brain tissues contributed to less sensitivity in *in vitro* fish gill cell bioassays.

Enhanced toxicity of domoic acid and alamethicin mixture was not observed in this study using an *in vitro* fish cell model. This is distinct from the *in vivo* Diptera larvae bioassay result by Ruiz *et al.* (2010). In contrast, Zhou et al. (2017) demonstrated consistent combined effects of mycotoxins AFB_1, DON and ZEN using *in vitro* fish cell model (BF-2) and *in vivo* zebra fish larvae. Their study revealed that binary combination of AFB_1 and DON, AF and ZEN resulted in synergism, DON and ZEN exhibited antagonism, and tertiary mixtures of AFB_1, DON and ZEN showed synergism to moderate antagonism depending on the concentration used (Zhou et al. 2017). Comparisons of the insignificant enhancement of domoic acid and alamethicin in the current study to the Diptera larvae results (Ruiz et al. 2010) can be tentative only, as different targets (*in vivo* vs *in vitro*), endpoints and exposure periods were used. The fact that domoic acid did not increase the alamethicin toxicity could also reflect the target specificity of the neurotoxin domoic acid.

3.4.3 Conclusion

The current study demonstrated that the tested mycotoxins exhibited cytotoxicity towards RTgill-W1 cells. This fish gill bioassay model suggest the potential of fish mortality from the marine mycotoxins, but only under conditions that release them in aqueous solution and bring them in contact with sensitive fish gills. The actual mycotoxin concentration in marine environments deserves further investigation. Significant cytotoxicity of major *A. sydowii* metabolites is especially concerning, as *A. sydowii* is often isolated from marine organisms and has previously caused sea fan coral mass mortality. The algal neurotoxins, saxitoxin and domoic acid, had no effect on fish gill cell viability. This is likely due to their specific modes of actions which target receptors in muscles and brains, but appear inactive in fish gill cells.

Chapter 4. Combined cytotoxicity of the phycotoxin okadaic acid and mycotoxins on intestinal and neuroblastoma human cell models*

Abstract

Mycotoxins are emerging toxins in the marine environment, which can co-occur with algal toxins to exert synergistic or antagonistic effects for human seafood consumption. The current study assesses the cytotoxicity of the algal toxin okadaic acid, shellfish, and dust storm-associated mycotoxins alone or in combination on human intestinal (HT-29) and neuroblastoma (SH-SY5Y) cell lines. Based on calculated IC_{50} (inhibitory concentration 50%) values, mycotoxins and the algal toxin on their own exhibited increased cytotoxicity in the order of sydowinin A < sydowinin B << patulin < alamethicin < sydowinol << gliotoxin ≈ okadaic acid against the HT-29 cell line, and sydowinin B < sydowinin A << alamethicin ≈ sydowinol < patulin, << gliotoxin < okadaic acid against the SH-SY5Y cell line. Combinations of okadaic acid–sydowinin A, –alamethicin, –patulin, and –gliotoxin exhibited antagonistic effects at low-moderate cytotoxicity, but became synergistic at high cytotoxicity, while okadaic acid–sydowinol displayed an antagonistic relationship against HT-29 cells. Furthermore, only okadaic acid–sydowinin A showed synergism, while okadaic acid–

*Chapter 4 was previously published as: Hayashi, A., Dorantes-Aranda,J.J., Bowman, J. P. and Hallegraeff, G., 2018. Combined cytotoxicity of the phycotoxin okadaic acid and mycotoxins on intestinal and neuroblastoma human cell models. *Toxins*, 10(12), p.526 with the exception that table 1 in the published article is excluded in this thesis chapter, figures 4.3, 4.4 & 4.5 appear in only the thesis chapter, and a few minor modifications in the main text.

sydowinol, –alamethicin, –patulin, and –gliotoxin combinations demonstrated antagonism against SH-SY5Y. While diarrhetic shellfish poisoning (DSP) from okadaic acid and analogues in many parts of the world is considered to be a comparatively minor seafood toxin syndrome, our human cell model studies suggest that synergisms with certain mycotoxins may aggravate human health impacts, depending on the concentrations. These findings highlight the issues of the shortcomings of current regulatory approaches, which do not regulate for mycotoxins in shellfish and treat seafood toxins as if they occur as single toxins.

4.1 Introduction

The importance of fungi in the marine environment has been increasingly recognised in recent years. They are capable of infecting a wide range of marine animals, including sea turtles (Sarmiento-Ramírez et al. 2010) and sea fan corals (Smith et al. 1996), and threatening human health through mycotoxin accumulation in seafood (Grovel, Pouchus & Verbist 2003). The majority of infectious fungi in the marine environment are considered to be of terrestrial origin (Pang et al. 2016), but atmospheric dust deposition and terrestrial runoff can facilitate the growth of fungi already residing in the marine environment and/or introduce them from terrestrial into marine environments. For example, an *Aspergillus sydowii* "bloom" (150,000 spores/m^2) along the east coast of Australia was observed after an extensive dust storm in 2009 (Hallegraeff et al. 2014). Similarly, increased dust deposition and nutrient input from terrestrial runoff is thought to have contributed to an outbreak of the fungal disease sea fan coral aspergillosis in the Caribbean (Harvell et al. 1999).

Fungal contaminants in seafood can also pose a significant human health risk. Several studies have shown that toxigenic fungal species can reside within the shellfish itself, seawater, and sediments from aquaculture farming areas. *Penicillium*, *Aspergillus*, *Trichoderma*, and *Cladosporium* have been isolated from such samples in France (Sallenave-Namont et al. 2000), Canada (Brewer, Greenwell & Taylor 1993), Algeria (Matallah-Boutiba et al. 2012), Russia (Zvereva & Vysotskaya 2005), Brazil (Santos et al. 2017), Italy (Greco et al. 2018), and Tunisia (Marrouchi et al. 2013). These genera of fungi are capable of producing toxic metabolites (mycotoxins), including aflatoxins (AF), zearalenone (ZEA),

deoxynivalenol (DON), fumonisins (FB), and ochratoxins (OTA) (Gonçalves, Naehrer & Santos 2016). These compounds exhibit a wide range of biological activities, including hepatocarcinogenic, genotoxic, carcinogenic, oestrogenic, nephrotoxic, and nephrocarcinogenic effects (Zain 2011). Evidence exists that some shellfish-associated fungal isolates were capable of producing highly toxic mycotoxins, such as gliotoxin by *Aspergillus fumigatus* (Grovel, Pouchus & Verbist 2003), patulin by *Penicillium* sp. (Vansteelandt et al. 2012), peptaibol by *Trichoderma* sp. (Poirier, Montagu, et al. 2007), and griseofulvin by *P. waksmanii* (Petit et al. 2004). These mycotoxins have been demonstrated to bio-accumulate in shellfish under both laboratory and natural conditions. A filtrate of marine-derived *T. koningii*, gliotoxin accumulated in shellfish (up to 2.9 µg/mg), and peptaibols were detected in shellfish and sediments (up to 5 ng/g from sediments) from aquaculture environments (Grovel, Pouchus & Verbist 2003; Poirier, Amiard, et al. 2007; Sallenave et al. 1999). C17-sphinganine analogue mycotoxin (C17-SAMT) was claimed to be solely responsible for high shellfish toxicity in Tunisia in 2006 (Marrouchi et al. 2013). Mycotoxins are now widely viewed as new emerging toxins in shellfish.

Mycotoxins on their own can pose a significant health risk for humans through shellfish consumption, but an even greater concern arises from their possible synergistic effects with co-occurring algal toxins. However, mycotoxins in shellfish are currently not monitored and information on the combined effects of algal toxins and mycotoxins is sparse. So far, an in vivo Diptera larval bioassay by Ruiz et al. has been the only study to assess the combined effects of the algal toxin domoic acid and mycotoxin. Their study revealed increased toxicity by up to 34.5 times (the synergism factor) when domoic acid and longibranchi-A-I were injected together into Diptera larvae (Ruiz et al. 2010). The proposed mechanism of this synergism was enhanced by an increase in Ca^{2+} influx into the cells by both domoic acid and novel peptaibol longibranchi-A-I (Ruiz et al. 2010).

The management of seafood safety is important for public health, market access, and public confidence. For example, a single incident of failure of detecting unacceptable levels of paralytic shellfish toxins (PST) in exported mussels resulted in AUD$24 million dollar economic loss to the Tasmanian seafood industry (Campbell et al. 2013). Current approaches to seafood safety management do not regulate for mycotoxins, and take no account of

combined effects of co-occurring seafood toxins and treat them as if they were to occur as individual compounds (Gonçalves et al. 2017; Stobo et al. 2008). In the worst-case scenario, we could experience unexpected fatal toxicity from enhanced effects from unregulated mycotoxins and algal toxins occurring below the regulatory limits. Therefore, the aim of this study was to identify the toxic interactions of major algal toxins (e.g., saxitoxin, domoic acid and okadaic acid) and shellfish-associated (e.g., gliotoxins, patulin and peptaibol) and dust-originated (*A. sydowii* metabolites and sterigmatocystin, see Chapter 2) mycotoxins (see Figure 3.1 and Table 3.1 in Chapter 3 for details of chemical structure and toxicological information) using human intestinal HT-29 and neuroblastoma SH-SY5Y cell line models. HT-29 and SH-SY5Y were chosen for assessing gastrointestinal and neurological effects, respectively, from saxitoxin (Gessner & Middaugh 1995), domoic acid (Teitelbaum et al. 1990) and okadaic acid (Valdiglesias et al. 2013). Toxin interactions such as synergisms, antagonism, and additive were quantitatively evaluated with the combination index (CI) method (Chou & Talalay 1984).

4.2 Materials and Methods

4.2.1 Cell line cultures

Human neuroblastoma SH-SY5Y was kindly provided by Ms Yilan Zhen and Dr. Kaylene Young (Menzies Institute for Medical Research, University of Tasmania, Australia). Human colorectal adenocarcinoma cells HT-29 were kindly provided by Dr. Anthony Baker (Tasmanian Institute of Agriculture, University of Tasmania and School of Land and Food, Australia). Both cell lines were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM, D0819, Sigma-Aldrich, Sydney, Australia) supplemented with 10% foetal bovine serum (FBS, Bovogen Biologicals, Melbourne, Australia), and 100 U/mL penicillin and 100 mg/mL streptomycin solution in a humidified incubator (5% CO_2, 37 °C). SH-SY5Y cells were routinely subcultured at a ratio of 1:30–1:50, and medium changeover occurred approximately every 5 d. HT-29 cells were routinely subcultured at a ratio of 1:3–1:8, and medium changeover occurred approximately every 4 d.

4.2.2 Mycotoxin and phycotoxin toxins

Four typical *Aspergillus sydowii* metabolite standards, sydowinin A, sydowinin B, sydowinol, and sydowic acid were kindly provided by Professor Hiromitsu Nakajima, Tottori University, Japan. These compounds were isolated from *A. sydowii* IFO 4284 and IFO 7531 strains. Full descriptions of UV, IR, and NMR spectra, chemical structures, and molecular weights of these metabolites were previously provided by Hamasaki, Sato & Hatsuda (1975a) & (1975b). The crystallised *A. sydowii* metabolites were weighted on a microbalance and dissolved in small volumes of acetone (>0.5 mL). Among the other fungal toxins tested, gliotoxin (G9893, Sigma-Aldrich) was dissolved in ethanol, alamethicin (A4665, Sigma-Aldrich) was dissolved in DMSO, and sterigmatocystin (S3255, Sigma-Aldrich) and patulin (P1639, Sigma-Aldrich) were dissolved in acetonitrile. Phycotoxin standards, saxitoxin (CRM-STX-f), domoic acid (CRM-DA-g), and okadaic acid (CRM-OA-d) were purchased from the National Research Council Canada. Concentrations used are expressed as µM.

4.2.3 Cytotoxicity bioassays

When cells reached >70% confluency, they were detached using a trypsin–EDTA solution. Detached cells were centrifuged 300 g for 5 min and resuspended. Cells were seeded to a 96-well plate at 1.0×10^4 cells/well for HT-29 and 3.0×10^4 cells/well for SH-SY5Y and allowed to attach for 24 h prior to toxin exposure. Each well contained 100 µL of cells suspension, and 0.5–3% (*v*/*v*) of algal toxin and mycotoxins stocks were added to the basal DMEM, which contained no supplemented FBS nor antibiotics. Concentration ranges of tested individual toxicity of algal toxins and mycotoxins were 1.33×10^{-9}–123.3 µM for SH-SY5Y, and 3.12×10^{-8}–235.6 µM for HT-29. For the combined cytotoxicity bioassay, the ranges were 0.019–214.9 µM for HT-29 and 0.016–169.6 µM for SH-SY5Y. Cells were rinsed once with DPBS (Dulbecco's phosphate-buffered saline, 0.9 mM $CaCl_2$; 0.50 mM $MgCl_2 \cdot 6H_2O$; 2.7 mM KCl; 1.5 mM KH_2PO_4; 137.9 mM NaCl; 8.1 mM $Na_2HPO_4 \cdot 7H_2O$). Toxin-containing DMEM was added to each well and incubated further for 24 h. Controls received only solvents, and the solvent concentration used in the assay was preliminary tested to have no significant effect on the cell viability compared to those received basal DMEM without solvents (data not shown). After toxin exposure, the cells were washed once again

with DPBS and 100 µL of the same basal media (without phenol red) containing 5% resazurin solution (O'Brien et al. 2000) were added to each well. Following additional 2 h incubation in the dark, the plate was read with a BMG FLUOstar OMEGA plate reader using excitation of 540 nm and emission of 590 nm. Cell viability was expressed as the percentage of fluorescence reading compared to the control (% of control). Four biological replicates (four wells) were prepared for each treatment.

4.2.4 Statistical analysis of cytotoxicity of individual mycotoxin and algal toxin

Data analysis was conducted with the decision tree proposed by Sérandour *et al.* (2012), except that in this experiment, the controls were preliminary tested to have no effect on cell viability and no further calculation was conducted when there was no bottom asymptote. Briefly, the dose response curves were fitted with the four-parameter logistic model (4PL), and 95% asymptotic confidence intervals were calculated using GraphPad Prism 7. The half-maximal inhibitory concentration (IC_{50}) indicating the concentration that caused a half-maximal viability was calculated for each toxin. IC_{50} was accepted if the fitting dose–response curve had $R^2 > 0.85$ and standard of error of log IC_{50} was <40%. One-way analysis of variance (ANOVA) was used to evaluate statistical differences between control and treatments. Tukey's honestly significant different (HSD) post hoc tests were performed when the main effect was significant. Appropriate data transformation was determined using Box–Cox transformation. ANOVA and follow-up statistical analyses were performed with the statistical software R (R Development Core Team, version 3.4.3). A significance level of 0.05 was applied in this study.

4.2.5 Median effect and combination index analysis of mycotoxin and algal toxin mixture

The cytotoxicity of mycotoxin and algal toxin mixture was analysed based on the Chou–Talalay method (Chou & Talalay 1984). The combination of mycotoxin and algal toxin were at an equipotency ratio (e.g., $(IC_{50})_1/(IC_{50})_2$ ratio) based on the calculated IC_{50} values using the graphpad prism 4PL model; therefore, each toxin roughly affects the cell

viability equally (Chou 2006). After determining the individual cytotoxicity, the binary mixture and individual toxin were tested against the cells in parallel. The dose–responses for individual toxins and their mixture were modelled using the median effect equation of the mass action law:

$$\frac{fa}{fu} = \left(\frac{D}{D_m}\right)^m$$

where *D* is the dose of the toxin, D_m is the median effect dose (e.g., IC_{50}), *fa* is the fraction affected by dose (*D*) (e.g., fractions of cell viability affected), *fu* represents the fraction unaffected, and *m* indicates the shape of the slope (*m* = 1, >1, and <1 indicate hyperbolic, sigmoidal, and flat sigmoidal curves, respectively). Toxin interactions were only analysed when the linear correlation coefficient (*r*) of the median effect plot was greater than 0.92.

The mycotoxin and algal toxins interaction was analysed by the combination index (CI) method derived from the median effect equation of the mass action law. The combination index was calculated using the following equation below (Chou 2006):

$$^n(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j}$$

where $^n(CI)_x$ is the combination index for n mycotoxins and algal toxins that inhibits x percent of a system (e.g., viability), $(D)_j$ are the doses that mixture of n phyco- and mycotoxins that inhibits x percent of a system, and $(D_x)_j$ are the doses that each phyco- and mycotoxin itself inhibits x percent of a system. CI < 1, = 1, and >1 indicate synergism, additive effect, or antagonism, respectively. CI values were calculated over a range of *fa* = 0.05 to 0.97 (5–97% toxicity). A confidence interval of 95% (95% CI) for CI was calculated based on sequential deletion analysis (SDA). The dose reduction index (DRI) values were determined for the combination that exhibited a synergistic relationship at IC_{25}, IC_{50}, IC_{75} and IC_{90}. DRI indicates the magnitude of how the dose of each drug in a mixture can be reduced at the given effect level compared to the doses of each drug alone. The dose–response analyses of toxin mixtures, CI, and DRI were performed with Compusyn software (ComboSyn Inc., Paramus, NJ, USA).

4.3 Results

4.3.1 Individual cytotoxicity of algal toxin and mycotoxin

The cytotoxicity of individual mycotoxins and phycotoxins on the human intestinal cell line HT-29 and human neuroblastoma cell line SH-SY5Y was evaluated using resazurine cell viability reagent. The tested mycotoxins, except sydowic acid, exhibited a dose-dependent effect with a range of inhibitory concentration 50% (IC_{50}) from 65 nM to 124 µM for HT-29, and from 45 nM to 144 µM for SH-SY5Y (Table 4.1, Figures 4.1 and 4.2). Micrographs of cells exposed to toxins displayed disoriented and smaller shapes with cell fragments, and loss of neurites (Figure 4.3).The tested concentration ranges of sydowic acid (HT-29: 0.028–283.75 µM, SH-SY5Y: 0.567–567.49 µM) showed no significant effect on viability for both HT-29 and SH-SY5Y (HT-29: $F(8,27) = 0.095$, $p = 0.999$, SH-SY5Y: $F(4,15) = 1.516$, $p = 0.248$). Sterigmatocystin reduced the viability of both HT-29 and SH-SY5Y in a dose-dependent manner with an incomplete sigmoid curve. The highest applicable concentration of 62 and 123 µM sterigmatocystin lowered the viability of HT-29 to 60%, and that of SH-SY5Y to 43%, respectively. Therefore, the IC_{50} of sterigmatocystin was not calculated. Okadaic acid displayed a dose-dependent effect on HT-29, with IC_{50} of 65 nM, and SH-SY5Y viability, with IC_{50} of 27 nM, whereas the other tested algal toxin, saxitoxin, and domoic acid had either no effect or minor effects on the viability of HT-29 and SH-SY5Y (maximum tested concentrations were 16.6–1.33 µM) (Figures 4.1 and 4.2). For the overall cytotoxicity ranking, based on the calculated IC_{50} values, the tested mycotoxin and algal toxin were found to be in the increasing order of sydowinin A < sydowinin B << patulin < alamethicin < sydowinol << gliotoxin ≈ okadaic acid in HT-29, and sydowinin B < sydowinin A << alamethicin ≈ sydowinol < patulin, <<gliotoxin < okadaic acid in SH-SY5Y.

Table 4.1 Summary of cytotoxicity of typical A. sydowii metabolites, dust storm/shellfish associated mycotoxins, okadaic acid, saxitoxin and domoic acid algal toxins on HT-29 and SH-SY5Y cells after 24h exposure. Inhibitory concentration 50% (IC50) values and 95% confidence interval (CI) were calculated from four biological replicates using 4PL model.

Toxin	HT-29 IC_{50} (µM)	95% CI	SH-SY5Y IC_{50} (µM)	95% CI
Typical A. sydowii metabolites				
Sydowinin A	124.30	113.60–136.00	117.80	105.60–131.40
Sydowinin B	93.06	82.20–105.40	143.8	116.00–178.20
Sydowinol	2.50	2.21–2.82	5.14	5.06–5.23
Sydowic acid	NE (283.75) [1]	-	NE (283.75) [1]	-
Dust storm/shellfish mycotoxins				
Sterigmatocystin	>61.67 [2]	-	~123.35 [2]	-
Patulin	17.46	10.79–28.28	2.23	2.15–2.32
Alamethicin	4.92	4.57–5.29	5.43	5.29–5.67
Gliotoxin	0.062	0.052–0.075	0.045	0.039–0.053
Algal toxins				
Okadaic acid	0.065	0.056–0.075	0.027	0.026–0.029
Saxitoxin	NE(3.32)[1]	-	NE (1.33)[1]	
Domoic acid	NE(16.60)[1]	-	>6.64[3]	

[1]NE indicates toxins had no significant effect within the tested concentration range. Numbers in brackets indicate the maximum applicable concentration tested. [2]The maximum applicable concentration of 61.67 µM and 123.35 µM sterigmatocystin lowered the viability to 59.54% on HT-29 and 42.88% on SH-SY5Y, respectively. [3]The maximum applicable concentration of 6.64 µM of domoic acid lowered the viability of SH-SY5Y to 78.8%.

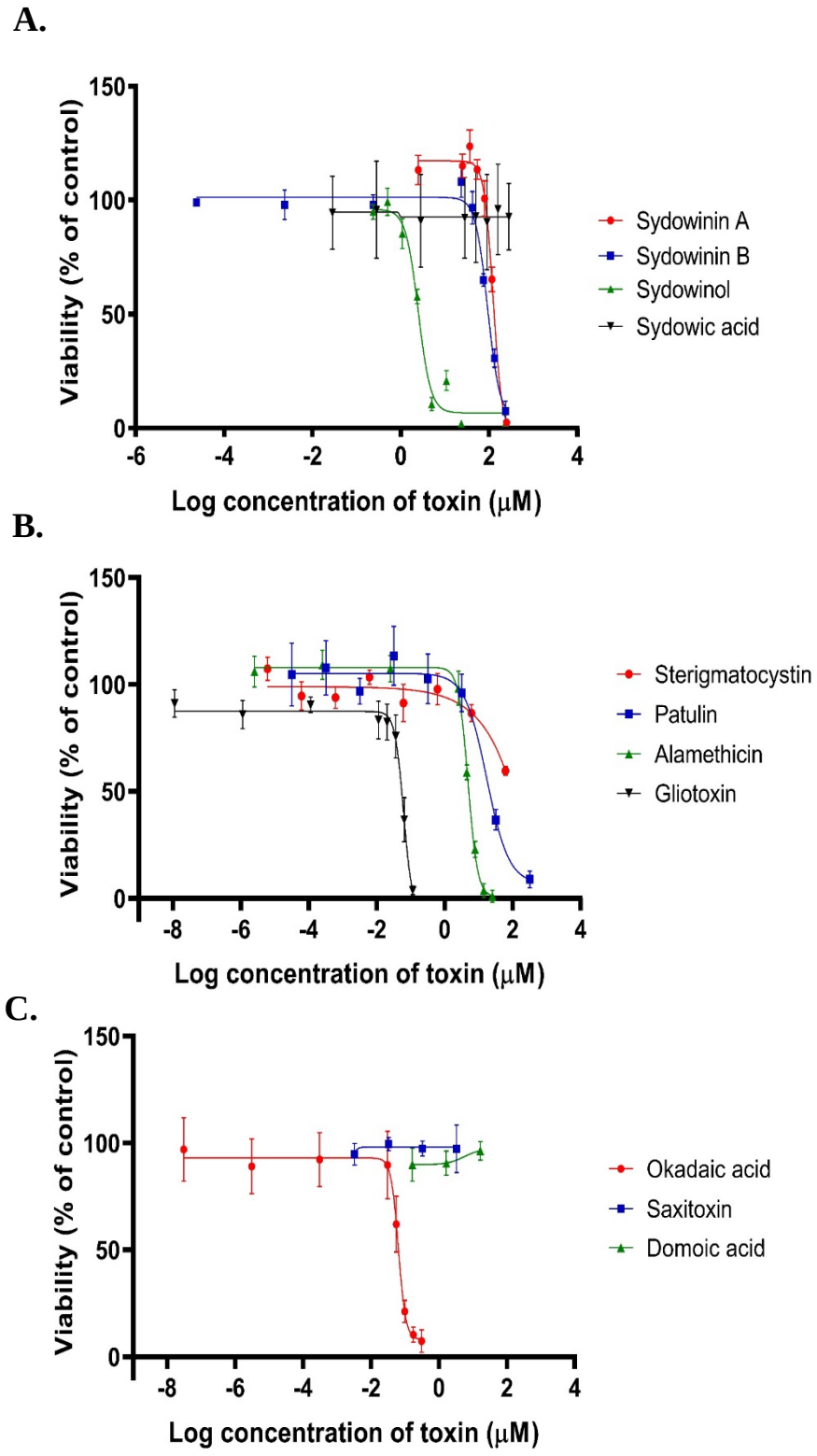


Figure 4.1 Dose-response curves of (A) major *A. sydowii* metabolites, (B) dust storm/shellfish associated mycotoxins (C) algal toxins on human intestinal HT-29 cells. Data are mean ± SD of four biological replicates.

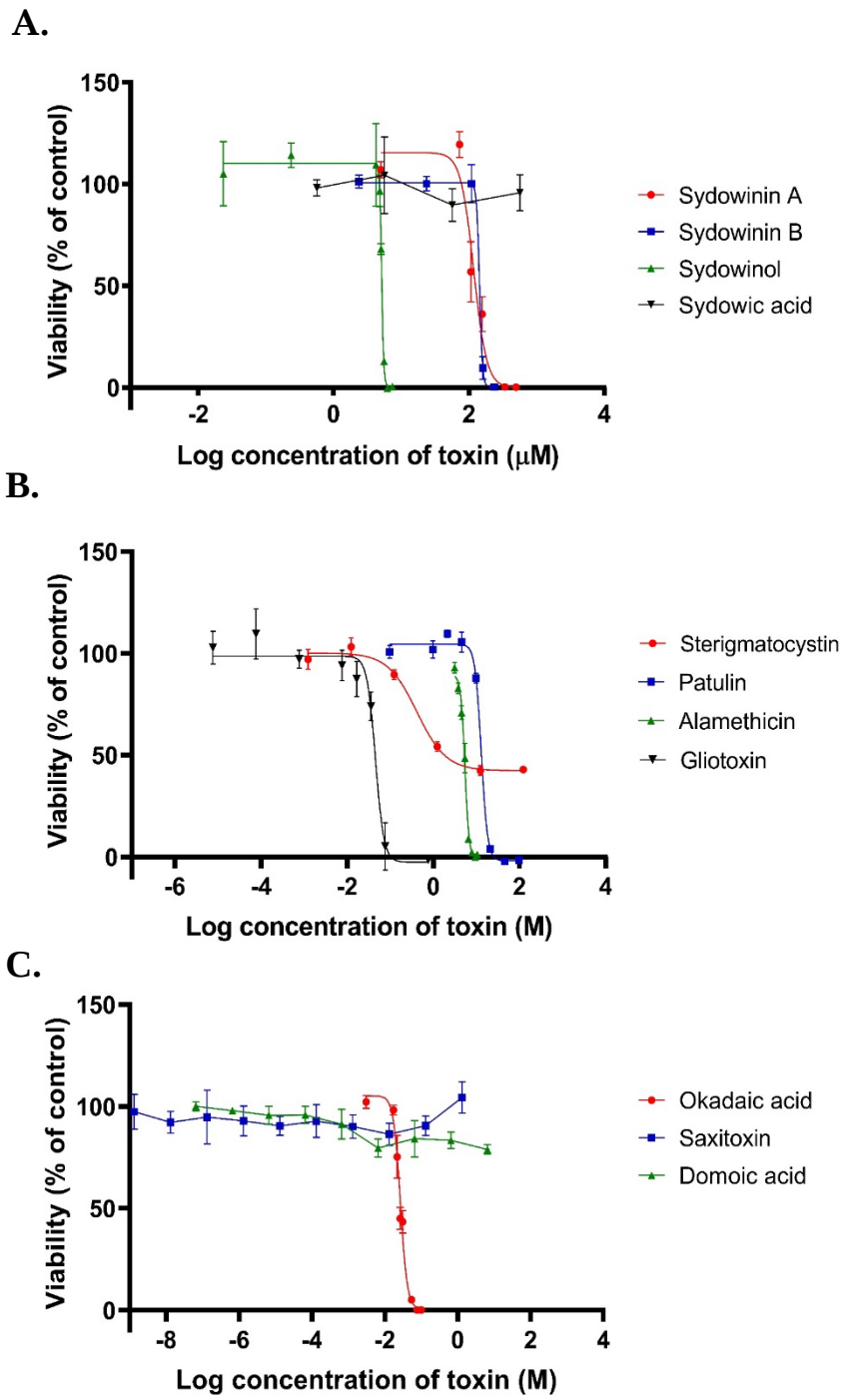
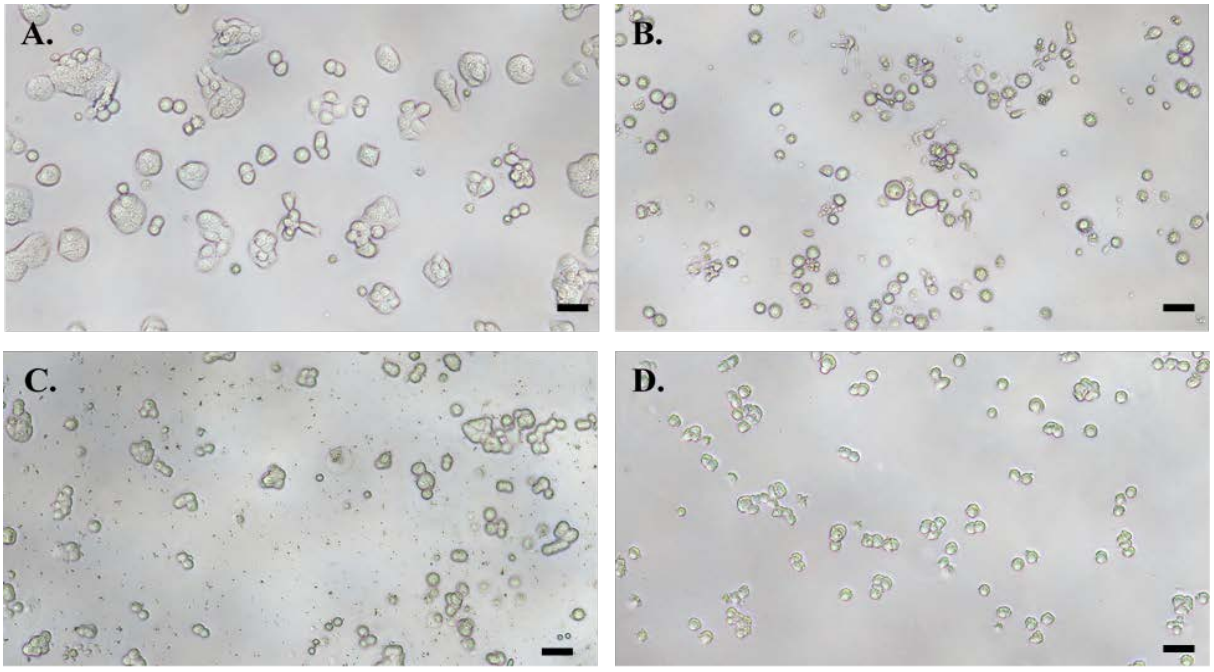


Figure 4.2 Dose-response curves of (A) major *A. sydowii* metabolites, (B) dust storm/shellfish associated mycotoxins (C) algal toxins on human neuroblastoma SH-SY5Y cells. Data are mean ± SD of four biological replicates.

HT-29



SH-SY5Y

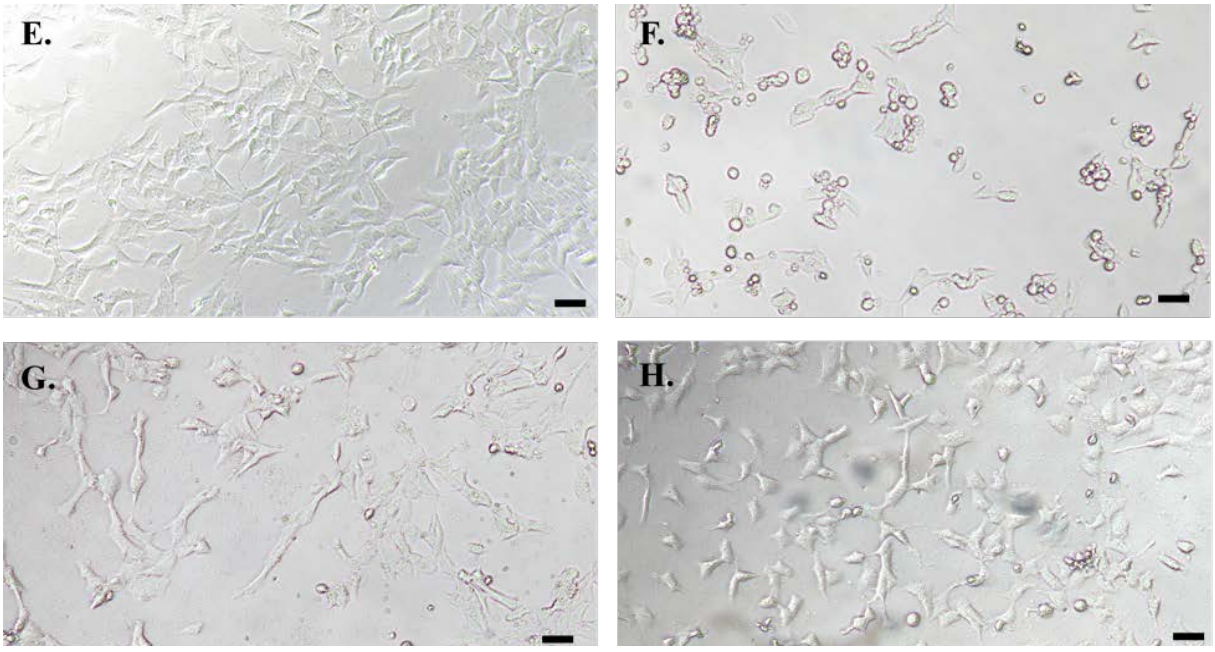


Figure 4.3 Micrographs of HT-29 (A,B,C,D) and SH-SY5Y (E,F,G,H) cells. Control cells exposed to basal medium (A,E) with normal spherical structures of HT-29, and with epithelial and fibroblastic structures. HT-29 cells exposed to (B) 0.13µM okadaic acid (C) 49 µM sydowinin A, (D) 69 µM patulin. Cells challenged with these toxins (B) –(D) exhibited disoriented and smaller shapes with cell fragments. SH-SY5Y cells exposed to (F) 0.047 µM okadaic acid (G) 88 µM sydowinin A (H) 3.2 µM patulin. Cells challenged with these toxins (F) – (H) exhibited loss of neurites, structure disorientation and cell fragments. Scale bars: 40 µM

4.3.2 Combined cytotoxicity of mycotoxins and algal toxin

Since okadaic acid was the only algal toxin which exhibited cytotoxicity on both HT-29 and SH-SY5Y cells, the effects of combined okadaic acid and mycotoxin sydowinin A, sydowinol, patulin, alamethicin, and gliotoxin on cell viability of HT-29 and SH-SY5Y were examined. Sydowinin B, sydowic acid, and sterigmatocystin were eliminated from the combined cytotoxicity assay because of their low cytotoxicity and limited solubility. Furthermore, the combination ratios were chosen to have an equipotent toxicity of each toxin (e.g., $(IC_{50})_1/(IC_{50})_2$ ratio) (Table 4.2), as there were no data available on the concentration of mycotoxins in shellfish under the natural settings, and this was recommended by Chou for an early stage study (Chou 2006). The combination index (CI) values were calculated from a fraction of cell viability affected (*fa*) values of 0.05 (corresponding to IC_{05}) to 0.97 (corresponding to IC_{97}), and the dose reduction index (DRI) was calculated when synergistic interactions were detected. All the binary mixtures of toxins showed a dose-dependent effect on HT-29 and SH-SY5Y cells (Figures 4.4 and 4.5).

Table 4.2 Molar combination ratio of okadaic acid and mycotoxin mixtures in the assay.

Toxin Mixture	Molar Combination Ratio	
	HT-29	SH-SY5Y
Okadaic acid:Sydowinin A	1:1925.0	1:14366.2
Okadaic acid:Sydowinol	1:38.7	1:190.7
Okadaic acid:Alamethicin	1:76.6	1:201.1
Okadaic acid:Patulin	1:270.4	1:82.6
Okadaic acid:Gliotoxin	1:1.04	1:1.68

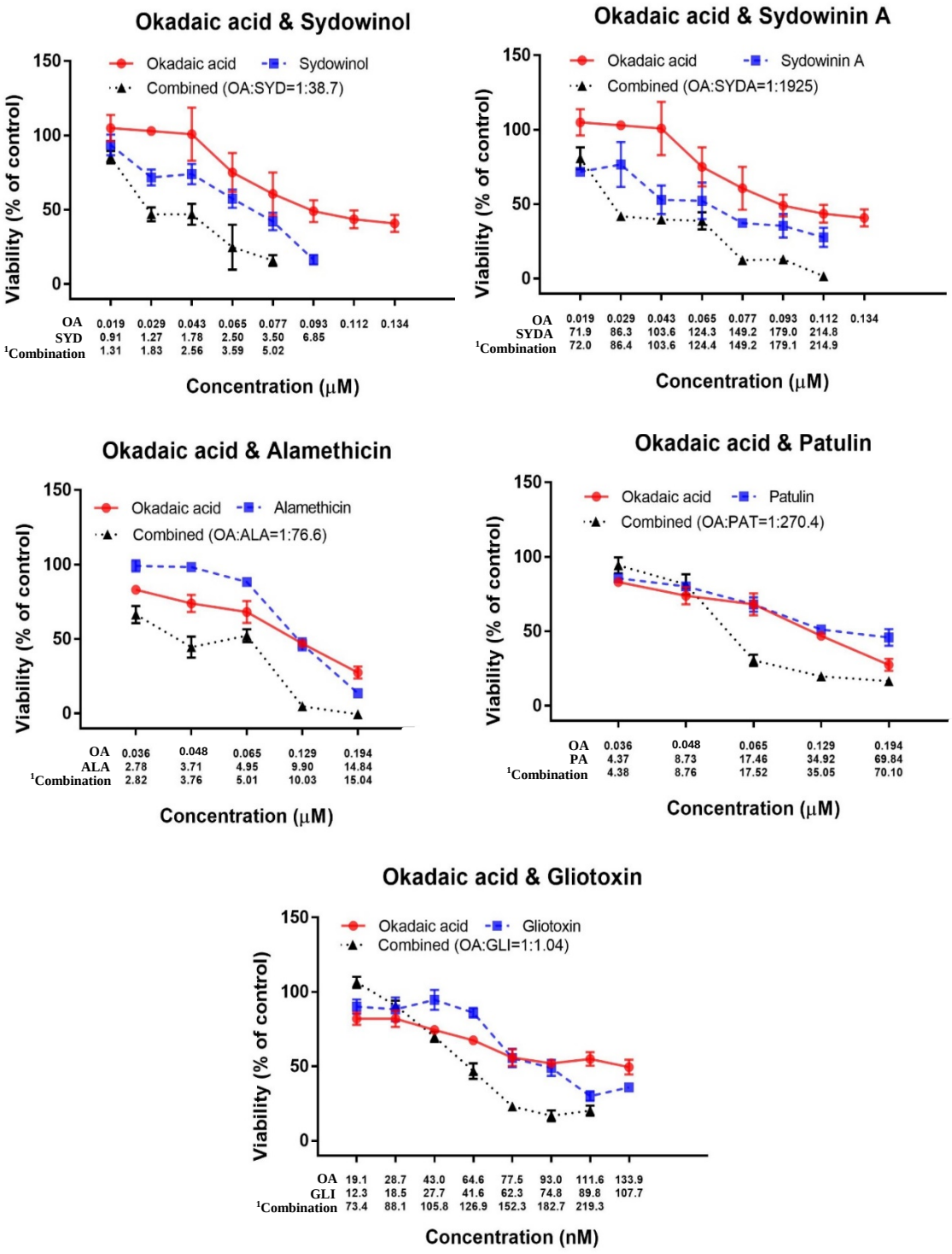


Figure 4.4 Okadaic acid (OA), sydowinol (SYD), sydowinin A (SYDA), alamethicin (ALA), patulin (PAT) and gliotoxin (GLI) and their binary mixture dose-responses for cytotoxicity against human intestine HT-29 cell line. [1]Concentrations in combinations were expressed as the sum of the concentrations of two toxins. Data are mean ± SD of four biological replicates.

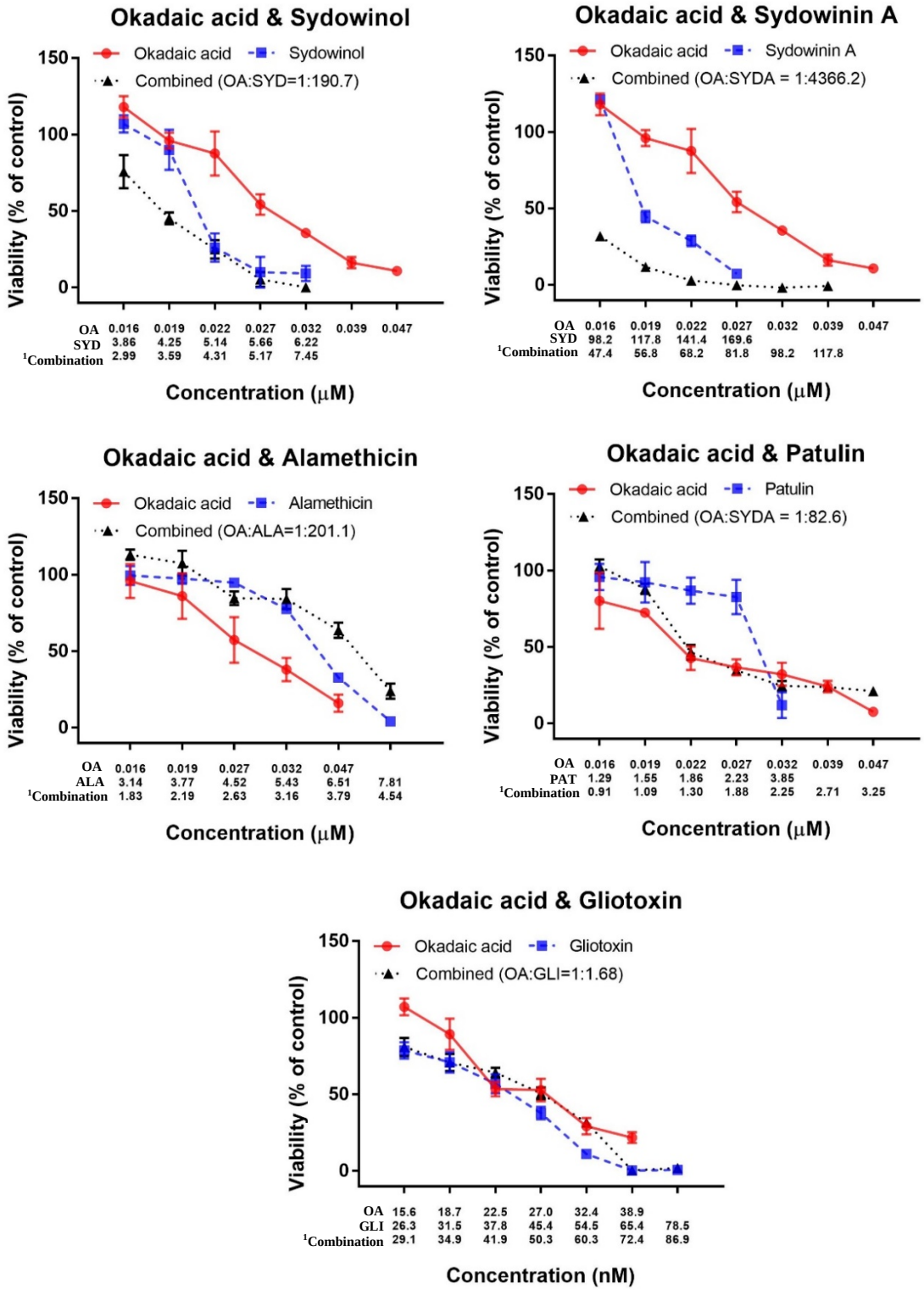


Figure 4.5 Okadaic acid (OA), sydowinol (SYD), sydowinin A (SYDA), alamethicin (ALA), patulin (PAT) and gliotoxin (GLI) and their binary mixture dose-responses for cytotoxicity against human neuroblastoma SH-SY5Y cell line. [1]Concentrations in combinations were expressed as the sum of the concentrations of two toxins. Data are mean ± SD of four biological replicates.

4.3.3 Okadaic acid and mycotoxins on human intestinal HT-29 cells

Okadaic acid–sydowinin A, –alamethicin, –patulin, and –gliotoxin binary mixtures displayed variations of the interaction types on human intestinal HT-29 cells dependent upon the effect levels (Figure 4.6). At low to moderate effect levels *(fa* < 0.65), these combinations exhibited antagonistic to additive effects, while they presented synergistic relationships at higher effect levels *(fa* > 0.65). In contrast to these okadaic acid–mycotoxin mixtures, okadaic acid–sydowinol mixtures displayed antagonistic effects at *fa* > 0.95 and a nearly additive interaction at *fa* < 0.95 (Figure 4.6). The DRI values for okadaic acid and mycotoxins varied from 1.8 to 12.5 and 1.8 to 12.2, respectively (Table 4.3). The greatest synergistic effect at *fa* = 0.9 was noted for the binary mixture of okadaic acid and gliotoxin, with a CI value of 0.41. For this combination, at the effect level of 0.9, the okadaic acid and gliotoxin mixture was 12.4 times more potent than okadaic acid alone, and 3 times more effective than gliotoxin alone.

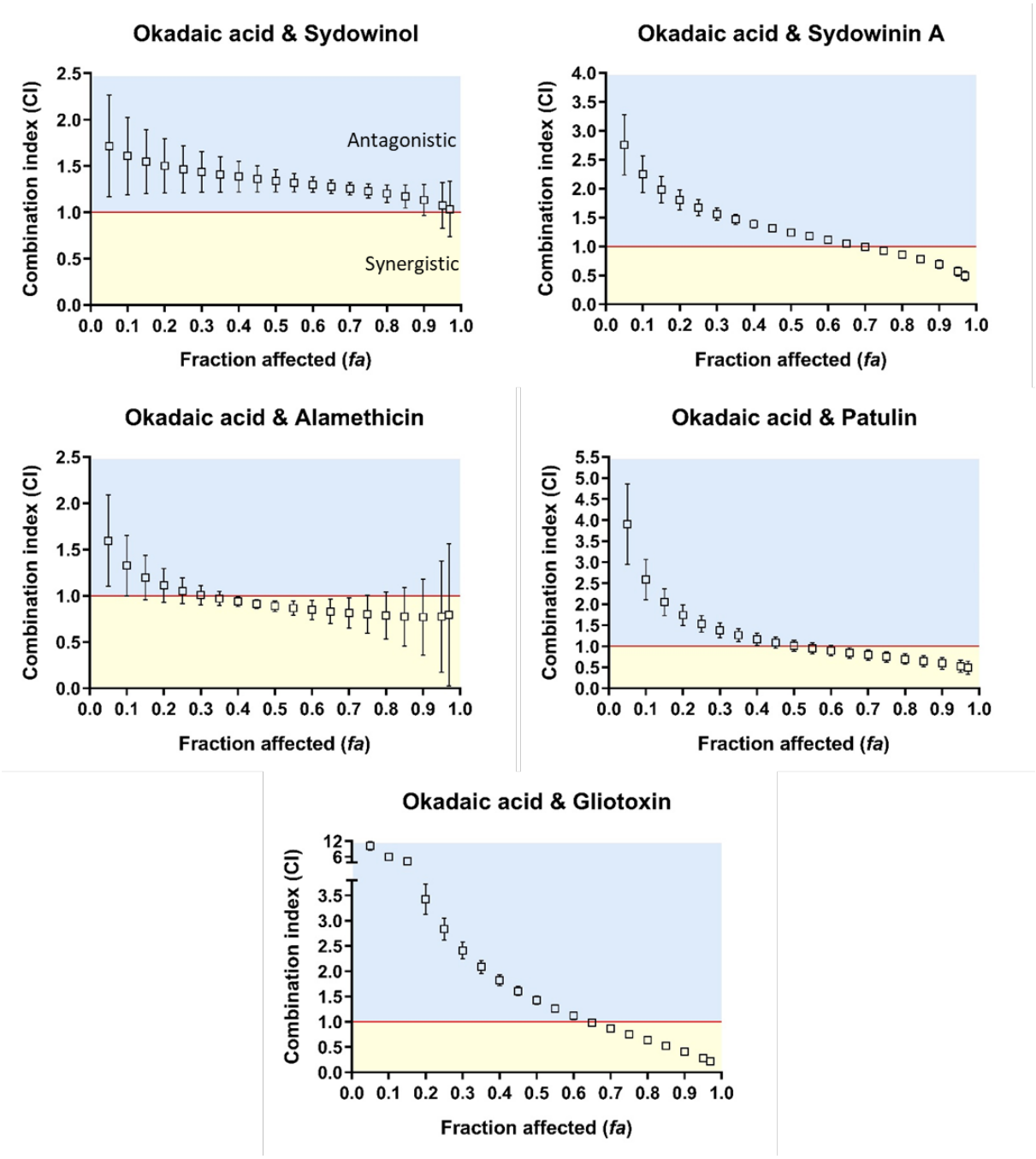


Figure 4.6 Combination index (CI)–fraction affected (*fa*, indicating fraction of cell viability affected. *fa* = 0.05–0.97 corresponds to 5–97% toxicity) curves for binary mixtures of okadaic acid and sydowinol, sydowinin A, alamethicin, patulin, and gliotoxin against human intestinal HT-29 cells. CI < 1, CI = 1, and CI > 1 indicate synergistic (orange rectangle), additive (red line), and antagonistic (blue rectangle) effects of binary mixtures, respectively. The error bar indicates 95% confidence intervals calculated using sequential deletion analysis (SDA).

Table 4.3 Combination index (CI) and dose reduction index (DRI) values for okadaic acid and mycotoxin combinations in HT-29 and SH-SY5Y cells at various effect levels (IC_{25}, IC_{50}, IC_{75} and IC_{90}). DRI values were only calculated when synergistic effects were detected. DRI implies fold of dose reduction for a given effect in a combination of toxins compared with the dose of each toxin alone.

Toxin Mixture	CI at				DRI at			
	IC_{25}	IC_{50}	IC_{75}	IC_{90}	IC_{25}	IC_{50}	IC_{75}	IC_{90}
HT-29								
Okadaic acid	1.67	1.24	0.93	0.69	-	-	2.72	3.61
Sydowinin A					-	-	1.80	2.43
Okadaic acid	1.47	1.34	1.23	1.13	-	-	-	-
Sydowinol					-	-	-	-
Okadaic acid	1.06	0.88	0.78	0.72	-	2.41	2.18	1.98
Alamethicin					-	2.14	3.14	4.61
Okadaic acid	1.53	1.01	0.75	0.53	-	-	1.76	1.95
Patulin					-	-	5.63	12.23
Okadaic acid	2.84	1.42	0.75	0.41	-	-	4.80	12.45
Gliotoxin					-	-	1.85	3.02
SH-SY5Y								
Okadaic acid	0.72	0.69	0.67	0.65	2.98	3.09	3.20	3.32
Sydowinin A					2.65	2.72	2.79	2.86
Okadaic acid	1.34	1.33	1.34	1.34	-	-	-	-
Sydowinol					-	-	-	-
Okadaic acid	1.30	1.33	1.36	1.41	-	-	-	-
Alamethicin					-	-	-	-
Okadaic acid	1.29	1.30	1.32	1.34	-	-	-	-
Patulin					-	-	-	-
Okadaic acid	1.30	1.48	1.68	1.91	-	-	-	-
Gliotoxin					-	-	-	-

4.3.4 Okadaic acid and mycotoxins on human neuroblastoma SH-SY5Y cells

Okadaic acid–sydowinol, –alamethicin, –patulin, and –gliotoxin mixtures on human neuroblastoma SH-SY5Y cells showed an antagonistic interaction type at all effect levels, except that at *fa* = 0.05; gliotoxin and okadaic acid exhibited an additive interaction type (Figure 4.7). The calculated CI values for these combinations varied from 1.15 to 2.21 (Figure 4.7). By contrast, okadaic acid–sydowinin A mixtures exhibited synergisms at all effect levels, with a CI of 0.65 at *fa* = 0.9. For this combination, at the effect level of 0.9, the okadaic acid and sydowinin A mixture was 3.3 times more effective than okadaic acid alone and 2.9 times more effective than sydowinin A alone (Table 4.3).

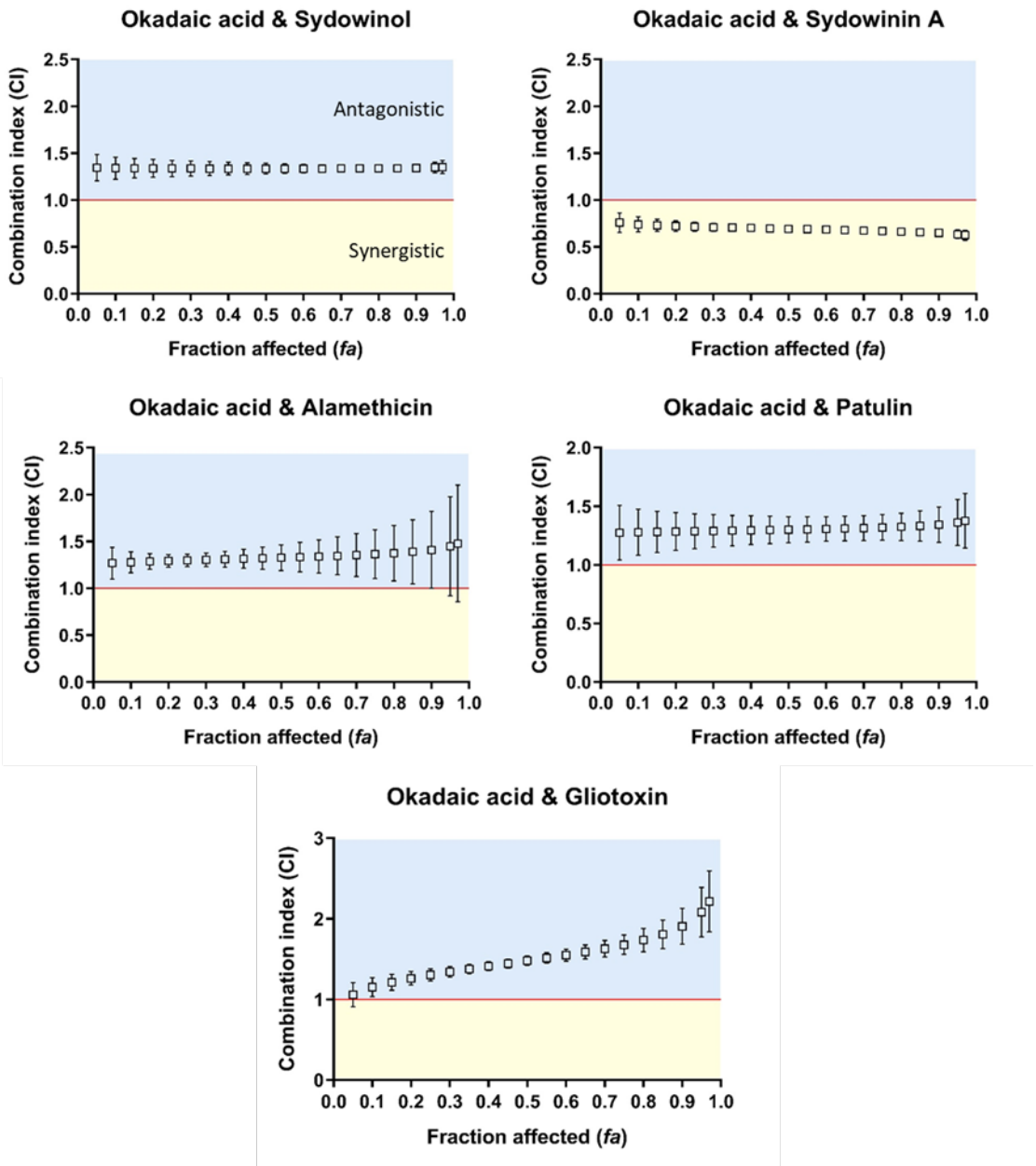


Figure 4.7 Combination index (CI)–fraction affected (*fa*, indicating fraction of cell viability affected. *fa* = 0.05–0.97 corresponds to 5–97% toxicity) curves for binary mixtures of okadaic acid and sydowinol, sydowinin A, alamethicin, patulin, and gliotoxin against human neuroblastoma SH-SY5Y cells. CI < 1, CI = 1, and CI > 1 indicate synergistic (orange rectangle), additive (red line), and antagonistic (blue rectangle) effects of binary mixtures, respectively. The error bar indicates 95% confidence intervals calculated using sequential deletion analysis (SDA).

4.4 Discussion

We demonstrated in this study that binary mixtures of the phycotoxin okadaic acid, and dust- and shellfish-associated mycotoxins exhibited cell line- and concentration-dependent antagonistic or synergistic interactions. Combinations of okadaic acid–sydowinin A, –alamethicin, –patulin, and –gliotoxin exhibited synergisms at higher effect levels and antagonisms at lower effect levels on HT-29. Interestingly, only okadaic acid–sydowinin A displayed synergism, whereas antagonism was noted for other combinations on SH-SY5Y at all effect levels. DRI values indicated that toxin doses can be theoretically reduced by up to 1.8 to 12-fold for the combination to have the same effect as that induced by each toxin on its own. These findings suggested that ingestion of a regulatory safe level of the algal toxin okadaic acid (0.16 mg OA equivalent/kg) could result in a health impact due to synergism with mycotoxin.

4.4.1 Synergisms between okadaic acid and mycotoxins

We speculate that synergistic effects of okadaic acid and the tested mycotoxins on HT-29 could be the result of the impairment of cell structure. Okadaic acid is the main lipophilic marine biotoxin produced by *Dinophysis* and *Prorocentrum* dinoflagellates and responsible for diarrhetic shellfish poisoning (DSP) in humans (Food and Agriculture Organization of the United Nations 2004). The reported concentration of okadaic acid in shellfish ranged from 0.007-0.267 mg OA equivalent/kg in the west coast of South Africa (Pitcher, Krock & Cembella 2011), 184-1,269 mg OA equivalent/kg in the western Mediterranean area (Bazzoni et al. 2018), 0.05-2.31 mg OA equivalent/kg in Portugal (Vale & Sampayo 2002). Okadaic acid is an inhibitor of serine/threonine protein phosphatases (PP), which affect various important cellular metabolic processes, leading to cytoskeleton and intestinal mucosa deterioration, digestive dysfunction, lipid metabolism disorders, oxidative stress, and cellular apoptosis (Wang et al. 2012). These series of events contribute to the gut barrier impairment and intestinal cell degeneration, which results in human diarrheic symptoms (Wang et al. 2012). The mycotoxin alamethicin, also known as peptaibol, forms pores in membranes, thereby increasing membrane permeability (Mueller & Rudin 1968).

Similarly, gliotoxin specifically binds to cytoplasmic membrane thiol groups, causing an increase in membrane permeability by affecting membrane protein orientation (Jones & Hancock 1988). Patulin also induces the depletion of nonprotein sulfhydryl groups and increases potassium efflux, which results in the loss of structural integrity of the plasma membrane (Riley & Showker 1991). While mycotoxins have different mechanisms of action, they all lead to a disruption of ion homeostasis and structural damage. This in turn potentially contributes downstream effects caused by okadaic acid in particular cytoskeleton deterioration, oxidative stress, and apoptosis. Furthermore, the observed shifts from antagonism to additive/synergism with increasing concentrations in the current study have also been reported in the similar study, where the interaction types of lipophilic phycotoxins (e.g., okadaic acid, pectenetoxin-2, yessotoxin, spirolide-1) were examined (Fessard et al. 2018).

Okadaic acid and sydowinin A exhibited synergistic effects on both the HT-29 and SH-SY5Y cell lines. Currently, we lack knowledge of the details of the mode of action of the major *Aspergillus sydowii* metabolites sydowinin A and sydowinol. Sydowinin A has been reported to have more potent immunosuppressive effects on the Con A-induced and lipopolysaccharide-induced proliferations of mouse splenic lymphocytes compared to other *A. sydowii* metabolites (Liu et al. 2016). The current study and other studies supported evidence of that the okadaic acid-induced PP inhibition also induces various neurotoxic effects (Arias et al. 1993; Tapia, Peña & Arias 1999). However, no major human neurotoxic symptoms from ingesting okadaic acid-contaminated seafood have been reported so far, probably due to the levels of okadaic acid accumulating more slowly in the brain compared to the stomach and gastrointestinal tract tissues (Matias, Traore & Creppy 1999). Synergistic relationships between okadaic acid and sydowinin A may have a basis in that cell injury caused by okadaic acid were further enhanced by the sydowinin A immune suppressive characteristics, but this requires investigation. The observed synergistic relationships with sydowinin A imply that even a low level of okadaic acid may cause significant neurotoxic effects in humans.

4.4.2 Antagonisms between okadaic acid and mycotoxin on SH-SY5Y

The combination of okadaic acid and the tested mycotoxins exhibited antagonistic relationships against SH-SY5Y neuroblastoma cells, whereas interactions were synergistic against HT-29 intestinal cell lines. Antagonistic interactions were also noted for HT-29 at the low effect level. The observed difference between two cell lines could be due to the different experimental conditions. These observed antagonisms could also be explained by multidrug resistance (MDR). MDR is regulated by P-glycoprotein (P-gp), which functions as an efflux transport pump, removing toxins from the plasma membrane, hence reducing cytotoxicity (Lum & Gosland 1995). Okadaic acid efflux occurred in okadaic acid-resistant Chinese hamster ovary cells with increased levels of P-gp, and human intestinal Caco-2 at low concentration (Ehlers et al. 2014; Tohda et al. 1994). Therefore, the observed antagonisms in SH-SY5Y cells could be related to less toxic mycotoxin binding to the target site, while okadaic acid is actively removed from the plasma membrane. This could lead to lower toxicity than estimated for the combined effect. This is supported by the fact that undifferentiated SH-SY5Y cells expressed some degree of P-gp expression, while HT-29 showed no detectable P-gp (Bates, Shieh & Tsokos 1991; Breuer et al. 1993). Furthermore, in the present study, mycotoxins were more abundant than okadaic acid in the binary mixtures, which could make mycotoxins more readily bind to the target site. Similarly, Alassane-Kpembi et al. (2015) suggested that the MDR drug efflux mechanism might explain the observed antagonism between deoxynivalenol (DON)–3-Acetyldeoxynivalenol (3-ADON) and DON–Fusarenon-X (FX) combinations. However, the suggested mechanisms of antagonisms remain speculative and require further study.

4.4.3 Conclusions

The present study demonstrated that binary mixtures of okadaic acid and shellfish- and dust-associated mycotoxins displayed cell line- and concentration-dependent interactions. The general interaction patterns observed in this study were a shift from antagonism to synergism with increasing concentrations on HT-29 cells, and antagonism or synergism at all concentrations on SH-SY5Y cells. The synergistic effects observed in the current study are of practical significance. While diarrhetic shellfish poisoning from okadaic acid and analogues

is widely considered to be a comparatively minor seafood toxin syndrome (e.g., no human fatalities have ever occurred), our human cell model studies provided preliminary insights that synergisms with mycotoxins can be expected to more seriously aggravate human health impacts.

This also suggests the need for implementing more studies of seafood where there is risk of the co-occurrence of mycotoxins and algal toxins. Our results clearly demonstrate that the toxin interaction type depends on the effect level and cell type. This points to difficulties of predicting toxin interactions from the known mechanisms of actions of individual toxins without actual experimental data (Chou 2006). Mycotoxins are emerging toxins in seafood, and their occurrence may increase due to increased terrestrial runoff, dust storms, and the use of mycotoxin contaminated aquaculture feeds (Gonçalves et al. 2017). The current study did not explore the precise cellular mechanisms behind the mycotoxin and algal toxin interaction, and suggested mechanisms therefore remain speculative, and deserve further study. Furthermore, as there exists no information on the mycotoxin concentration in shellfish *in situ*, the present study employed equipotent ratio as suggested by Chou for an early stage study (Chou 2006) which might not represent the true exposure scenario. Therefore, future work should prioritise exploring levels of mycotoxin accumulation in shellfish, and determining the interaction types of commonly occurring algal toxins (e.g., saxitoxin and domoic acid), and other mycotoxins (e.g., DON, AF, ZEA, FB, and OTA) (Chou 2010). Multiple mixtures (e.g., more than two toxins) should also be considered. Our results highlight the possible risks of toxin co-occurrence in seafood, a scenario which is not considered in current shellfish safety regulations.

Chapter 5. Conclusions and future directions

Climate change in Australia has the potential to significantly alter connectivity between terrestrial and marine ecosystems, and thereby impact on seafood security and safety. Increased loadings of nutrients and terrestrial microbes from dust storms and land-runoff may increase marine ecosystem impacts from fungal pathogens, adding to those from algal blooms. The scenario of a mycotoxin-producing fungal bloom being delivered into coastal shellfish farms simultaneously exposed to toxin-producing algae, as suggested by events in Canada in 1987 and France in 1990, deserves critical attention, as does the potential of synergism, antagonism and additivity by commonly co-occurring phycotoxins. The present body of work confirmed that the 2009 Australian dust storm generated Aspergillus mycotoxins and shellfish associated mycotoxins with strong cytotoxicity against fish gill cells. Furthermore, these mycotoxins exhibited the capability of increasing cytotoxicity from dinoflagellate okadaic acid by 1.8 to 12 fold. Interactions between Australian marine mycotoxins and saxitoxins and domoic acid remain to be further defined.

5.1 *Aspergillus sydowii* studies

Dust originated (ASBS), terrestrial (FRR5068) and sea fan aspergillosis pathogenic (FK1) *Aspergillus sydowii* strains studied here exhibited the similar production of major metabolites such as sydowinin A, sydowinin B, hydroxysydonic acid, sydowinol, sydonic acid and sydonol [Chapter 2]. Other minor metabolites of these isolates varied mong the strains. Further isolation of fungal cultures from the 2009 dust storm CPR silks (Hallegraeff et al. 2014) confirmed the dominance of *A. sydowii* (73.7%) but also revealed varying metabolite profiles which all included sydonic acid. Other minor fungal species were also found such as *Cladosporium, Penicillium, Aspergillus* and unknown species (based on metabolite analysis), suggesting the possible secondary colonisation of the 2009 *A. sydowii* fungal rafts.

Crude extracts of both terrestrial (FRR5152) and marine *A. sydowii* (ASBS) exhibited no or minor effects on photophysiological performance (maximum quantum yield, F_v/F_m) of clade A1 and clade C *Symbiodinium* dinoflagellates, respectively [Chapter 2]. The major *A. sydowii* metabolites (including sydowinin A, sydowinin B, sydowinol and sydowic acid) reduced photophysiological performance (F_v/F_m) of the coral dinoflagellate endosymbiont *Symbiodinium*. The *Symbiodinium* clade A and C strains displayed moderate (up to 24% reduction compared to control) and high (up to 41% reduction) sensitivities, respectively, while clade A1 exhibited lower sensitivity. These observed differences in sensitivity match sensitivities to coral bleaching (Toller, Rowan & Knowlton 2001) and may reflect broader differences in disease susceptibility.

Further studies are required, including: characterisation of novel strain-specific *A. sydowii* metabolites, examination of their possible role in pathogenicity of sea fan aspergillosis, and examination of the impacts of *A. sydowii* metabolites on the symbiosis between cnidarian hosts and endosymbiont *Symbiodinium* for example using the sea anemone *Aiptasia* as a model (Chakravarti & van Oppen 2018).

5.2 General impacts of fungi on marine animals using a fish gill cell line model

Shellfish and dust associated mycotoxins exhibited cytotoxicity against the fish gill RTgill-W1 cells in the order of sydowinin A << sydowic acid < patulin < sydowinol < alamethicin <<gliotoxin, based on the calculated toxicity (IC_{50}, inhibitory concentration 50%) [Chapter 3]. Other tested mycotoxins sydowinin B and sterigmatocystin, and the algal toxins saxitoxin and domoic acid exhibited no effect on fish gill cell viability. Unlike mycotoxins, algal toxins have more specific modes of actions which target receptors in muscles and brains, hence these specificities contributed their inactivity in fish gill cells. The applicability of the fish gill bioassay for mycotoxins is well demonstrated in this study. Although we lack toxicity information of the tested mycotoxins on whole fish, the present experimental data suggest the potential of fish mortality by mycotoxins especially if they reach sufficiently high

aqueous concentrations. Further research on the mycotoxin concentration level in the real situation would deepen the understanding of their effects on marine organisms. The previously reported synergism between domoic acid and peptaibol by Ruiz et al. (2009) using a Diptera larvae bioassay was not observed in the current bioassay using RTgill-W1. Comparisons of the insignificant potentiation of domoic acid and alamethicin in the current study to the Diptera larvae results are tentative only, as different targets, endpoints and exposure periods were used. The different results most likely also reflect the target specificity of the neurotoxin domoic acid. The precise mechanisms underpinning these contradictory results warrant follow-up investigations.

5.3 Combined effects of algal and fungal toxins

Single and combined toxicities of the major algal toxins, shellfish- and dust storm-associated mycotoxins were examined against human intestinal HT-29 and neuroblastoma SH-SY5Y cell lines [Chapter 4]. Based on the calculated IC_{50} (inhibitory concentration 50%), the cytotoxicity order of single toxin on HT-29 and SH-SY5Y cells were sydowinin A < sydowinin B << patulin < alamethicin < sydowinol << gliotoxin ≈ okadaic acid, and sydowinin B < sydowinin A << alamethicin ≈ sydowinol < patulin, << gliotoxin < okadaic acid, respectively. Furthermore, the algal toxins saxitoxin and domoic acid exhibited either no cytotoxicity or minor cytotoxicity to both HT-29 and SH-SY5Y. Using the combination index method by Chou and Talalay (1984), combinations of okadaic acid–sydowinin A, –alamethicin, –patulin, and –gliotoxin demonstrated antagonistic to synergistic effects on HT-29 cells with increasing cytotoxicity, whereas okadaic acid–sydowinol displayed an antagonistic relationship against HT-29 cells at all cytotoxicity levels. Furthermore, binary mixtures of okadaic acid–sydowinin A showed synergisms on SH-SY5Y cells. However, other tested combinations of sydowinol, –alamethicin, –patulin, and –gliotoxin demonstrated antagonism against SH-SY5Y cells. Toxin interactions at IC_{90} are summarised in Figure 5.1. DRI (dose reduction index) values suggested that in combination, each toxin doses could be reduced by 1.8 to 12-fold to have the same effect as that induced by each individual toxin on its own. The observed synergistic effects imply that intake of a regulatory safe concentration

of okadaic acid (0.16 mg OA equivalent/kg) could aggravate human health impacts from the synergistic effects from co-occurring mycotoxins.

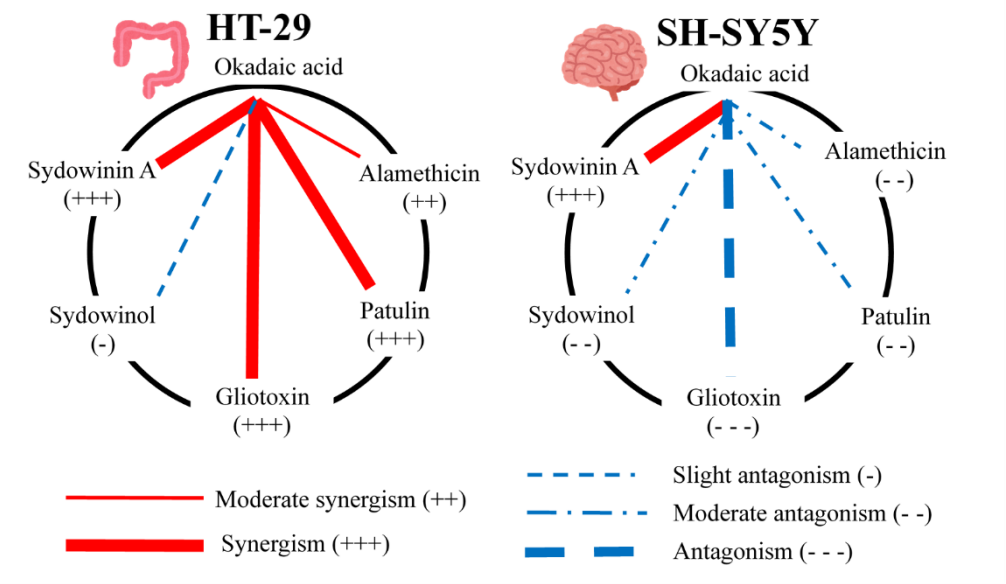


Figure 5.1 Polygonograms of toxin interaction types between mycotoxins and okadaic acid at IC_{90} against HT-29 human intestinal (left) and SH-SY5Y (right) human neuroblastoma cell lines. Solid line indicates synergism, dashed line indicates antagonism.

Future studies need to prioritise examining the interaction types of commonly occurring algal toxins (e.g., saxitoxin and domoic acid) and other mycotoxins (e.g., deoxynivalenol, aflatoxin, zearalenone, fumonisins and ochratoxins). Type of mycotoxins, concentration levels and proportions in which they co-occur with phycotoxins would also require further investigation. However, a major obstacle of assessing combined toxicity of saxitoxin and domoic acid is that neuroblastoma cell lines exhibited low sensitivities to these toxins (LePage *et al.*, 2005; Cañete and Diogène, 2008, Chapter 4). These insensitivities are most likely due to their specific mode of actions, and lack of the target glutamate receptors (LePage et al. 2005) and action potentials (Kogure et al. 1988). A pilot study demonstrated that it is possible to increase sensitivity by differentiating SH-SY5Y cells which allows them to develop extensions of neuritic processes (Påhlman et al. 1981), increase electrical excitability of the plasma membrane (Åkerman, Scott & Andersson 1984; Kafitz et al. 1999) and synaptic vesicle recycling (Sarkanen et al. 2007), activate neurotransmitter (Lopes et al.

2010), and neurotransmitter receptors (Adem et al. 1987). The results obtained showed minor increases in sensitivity of differentiated cells to saxitoxin and domoic acid with a ~20% reduction in viability compared to undifferentiated cells (Figure 5.2). Quantitative analysis of toxin combinations require complete dose response curves (0 – 100 % responses), and hence the observed low sensitivities by neuroblastoma cells would limit future investigation of other combinations of saxitoxin and domoic acid. In contrast to immortalised cell lines, primary cell cultures and organoids retain the functional characteristics of the original tissue such as the presence of the domoic acid target NMDAR (N-methyl-d-aspartate receptors), and saxitoxin target voltage gated sodium channels (Lancaster et al. 2013; LePage et al. 2005; Yakoub & Sadek 2018). Therefore, it is recommended that future studies on combined effects of fungal and algal toxins should focus on development of *in vitro* bioassay using more representative primary cell or organoid models such as cardiomyocytes for saxitoxin and domoic acid testing.

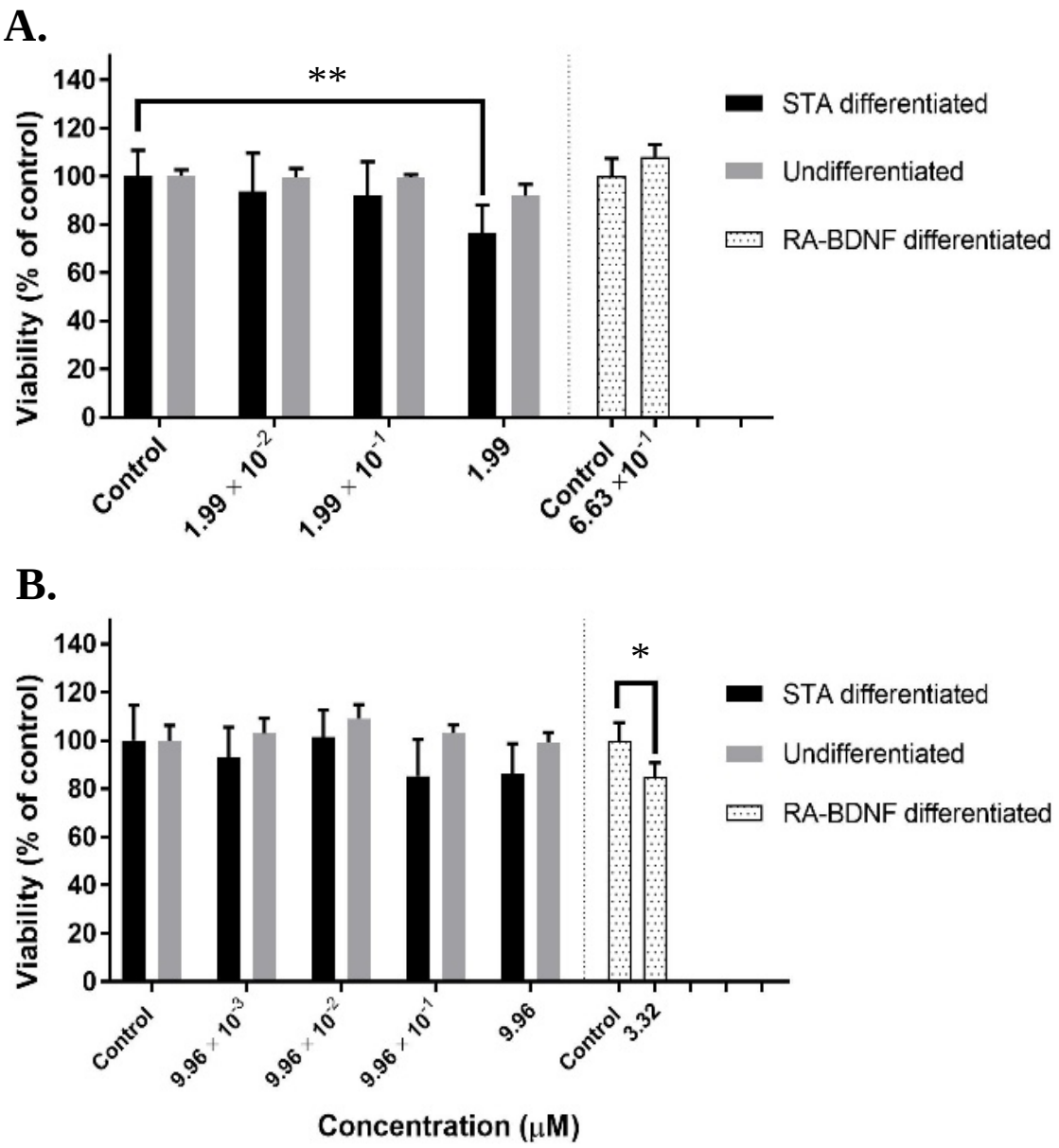


Figure 5.2 Comparison of SH-SY5Y human neuroblastoma sensitivity to saxitoxin (A) and domoic acid (B) between STA (staurosporine), RA (retinoic acid)-BDNF (brain-derived neurotrophic factor) differentiated and undifferentiated cell lines. Details of differentiation protocol and relevant materials are in Appendix A.1. Results are expressed as % viability compared to control (mean ± SD from four biological replicates). ANOVA was performed to determine statistical difference in viability between control, STA differentiated and undifferentiated treatments. Student t-test was performed to determine statistical significance between treatment and control (***$p < 0.001$ **$p < 0.01$, *$p < 0.05$).

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Appendix A

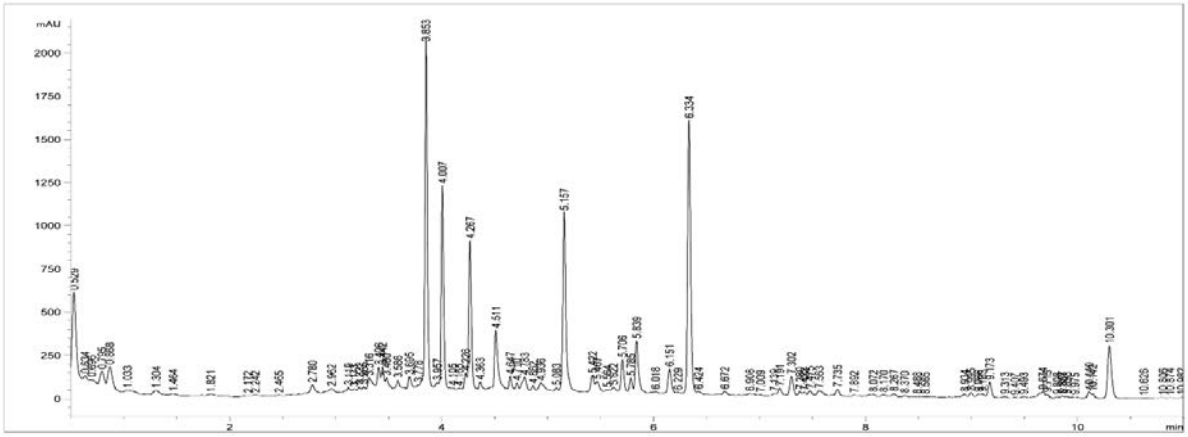
A.1 Supplementary data for Chapter 2

Table A.1.1 *Aspergillus sydowii* isolates examined in this study.

ID	Source	Location
FRR34	Wheat or flour sample	Not available
FRR2972	Dried fish; *Puntius javanicus*	Kalibaru, Indonesia
FRR2991	Dried fish; *Sardinella fimbriata*	Bogor, Indonesia
FRR4822	Partially dried sultana grapes	Mildura, VIC, Australia
FRR5068	Air, wardroom duct	Naval vessel
FRR5136	Water bath with ethylene glycol	Maribyrnong, VIC, Australia
FRR5152	Water bath with ethylene glycol additive	Maribyrnong, VIC, Australia
ASBS[1]	CPR silk after dust storm in 2009	Between Brisbane and Sydney, Australia
FK1	Diseased *Gorgonia ventalina*	Key West, Florida

[1] defined as the original *A. sydowii* culture isolated in 2009.

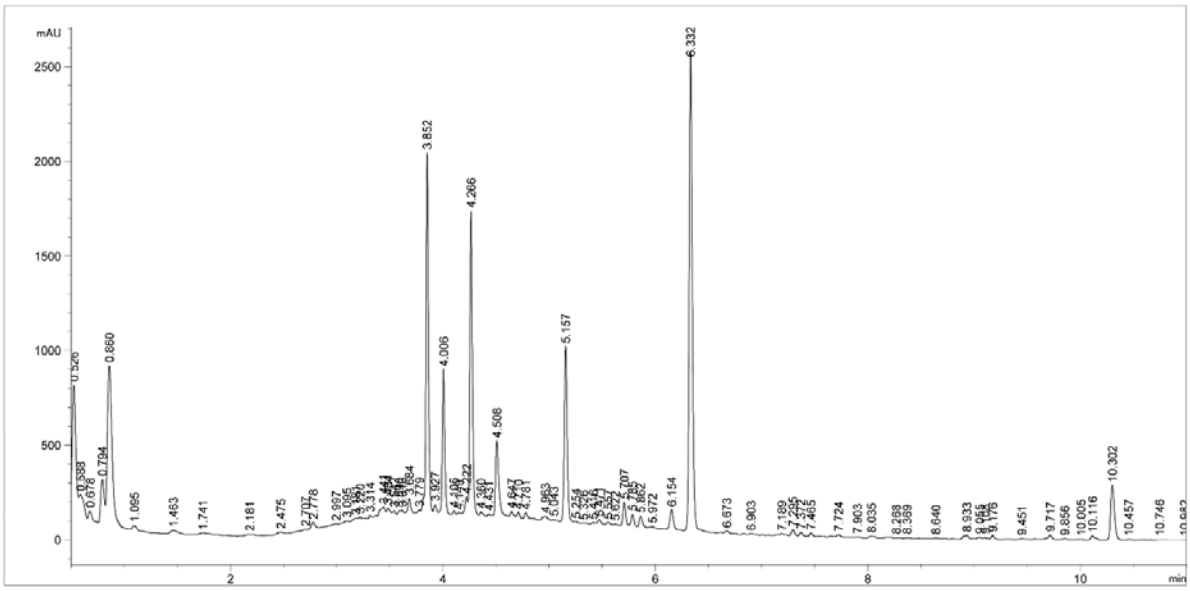
Table A.1.2 HPLC/DAD profile of *A. sydowii* FRR5068.



Peak Number	Ret. Time	Area	% Area	Cumulative % Area	Peak Number	Ret. Time	Area	% Area	Cumulative % Area
1	0.53	1,273	6.61	6.61	45	5.62	40	0.21	66.61
2	0.63	34	0.17	6.78	46	5.71	331	1.72	68.33
3	0.70	21	0.11	6.89	47	5.79	99	0.51	68.84
4	0.79	157	0.82	7.71	48	5.84	542	2.81	71.65
5	0.87	290	1.51	9.21	49	6.02	15	0.08	71.73
6	1.03	76	0.39	9.61	50	6.15	306	1.59	73.32
7	1.30	91	0.47	10.08	51	6.23	5	0.03	73.35
8	1.46	31	0.16	10.24	52	6.33	3,078	15.97	89.32
9	1.82	34	0.18	10.42	53	6.42	14	0.07	89.39
10	2.17	7	0.03	10.45	54	6.67	68	0.35	89.75
11	2.24	11	0.06	10.51	55	6.91	8	0.04	89.79
12	2.46	8	0.04	10.55	56	7.01	8	0.04	89.83
13	2.78	161	0.84	11.39	57	7.14	9	0.05	89.88
14	2.96	98	0.51	11.89	58	7.19	64	0.33	90.21
15	3.12	27	0.14	12.04	59	7.30	236	1.22	91.43
16	3.17	8	0.04	12.08	60	7.38	21	0.11	91.54
17	3.22	21	0.11	12.19	61	7.42	10	0.05	91.59
18	3.26	18	0.09	12.28	62	7.48	38	0.20	91.79
19	3.32	131	0.68	12.96	63	7.56	88	0.46	92.25
20	3.41	98	0.51	13.47	64	7.73	73	0.38	92.63
21	3.44	85	0.44	13.90	65	7.89	8	0.04	92.67
22	3.48	36	0.19	14.09	66	8.07	39	0.20	92.87
23	3.59	98	0.51	14.60	67	8.17	16	0.09	92.96
24	3.69	124	0.64	15.24	68	8.27	27	0.14	93.10
25	3.78	6	0.03	15.27	69	8.37	17	0.09	93.19
26	3.85	3,143	16.31	31.58	70	8.49	7	0.04	93.22
27	3.96	12	0.06	31.64	71	8.56	7	0.04	93.26
28	4.01	1,760	9.13	40.77	72	8.93	30	0.16	93.42
29	4.11	6	0.03	40.80	73	9.00	39	0.20	93.62
30	4.17	9	0.05	40.85	74	9.07	9	0.05	93.67
31	4.23	51	0.26	41.11	75	9.10	9	0.05	93.71
32	4.27	1,324	6.87	47.98	76	9.17	170	0.88	94.60
33	4.36	68	0.35	48.34	77	9.31	20	0.11	94.70
34	4.51	701	3.64	51.97	78	9.41	6	0.03	94.74
35	4.65	111	0.58	52.55	79	9.49	19	0.10	94.83
36	4.71	44	0.23	52.78	80	9.67	75	0.39	95.22

37	4.78	172	0.89	53.67	81	9.72	12	0.06	95.28
38	4.86	35	0.18	53.86	82	9.81	7	0.04	95.31
39	4.94	144	0.75	54.60	83	9.86	12	0.06	95.37
40	5.08	21	0.11	54.71	84	9.90	6	0.03	95.40
41	5.16	2,072	10.75	65.47	85	9.97	11	0.06	95.46
42	5.42	119	0.62	66.09	86	10.11	33	0.17	95.63
43	5.47	53	0.27	66.36	87	10.14	9	0.05	95.68
44	5.56	9	0.04	66.40	88	10.30	779	4.04	99.72

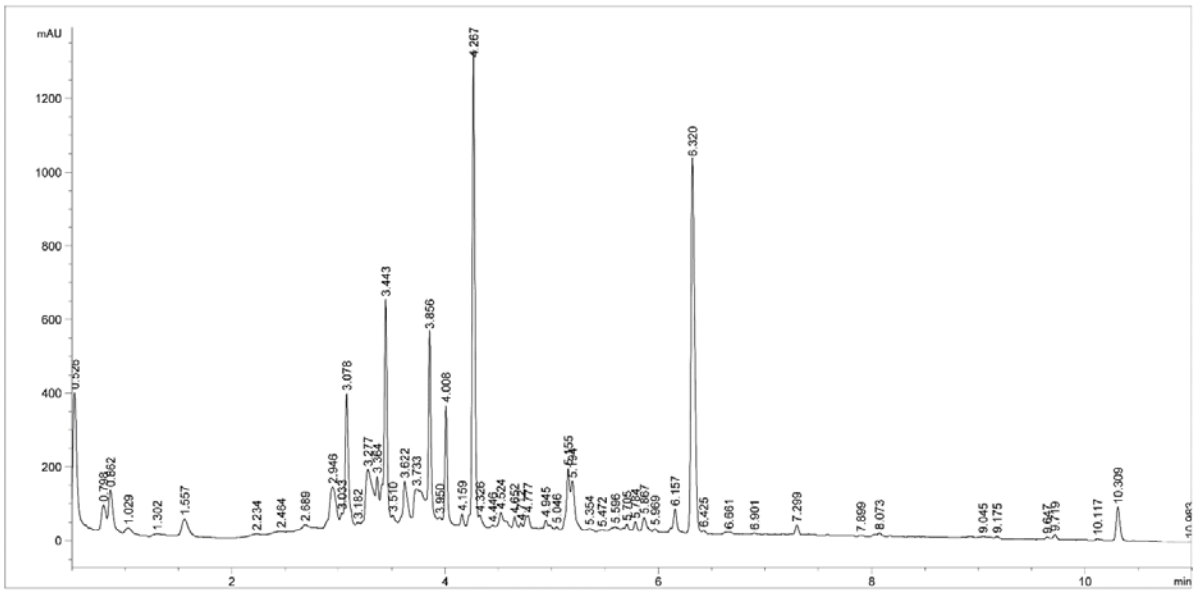
Table A.1.3 HPLC/DAD profile of *A. sydowii* ASBS.



Peak Number	Ret. Time	Area	% Area	Cumulative % Area	Peak Number	Ret. Time	Area	% Area	Cumulative % Area
1	0.53	1,309	6.09	6.09	40	5.16	1,710	7.96	66.25
2	0.59	111	0.51	6.61	41	5.25	6	0.03	66.27
3	0.68	83	0.39	7.00	42	5.33	9	0.04	66.32
4	0.79	343	1.60	8.59	43	5.42	10	0.05	66.36
5	0.86	2,109	9.82	18.41	44	5.47	40	0.19	66.55
6	1.09	65	0.30	18.71	45	5.56	25	0.12	66.67
7	1.46	88	0.41	19.12	46	5.62	9	0.04	66.71
8	1.74	68	0.31	19.44	47	5.71	212	0.99	67.70
9	2.19	32	0.15	19.59	48	5.79	104	0.48	68.18
10	2.48	32	0.15	19.74	49	5.86	114	0.53	68.71
11	2.71	6	0.03	19.76	50	5.97	19	0.09	68.80
12	2.78	79	0.37	20.13	51	6.15	233	1.08	69.88
13	3.00	12	0.06	20.19	52	6.33	5,152	23.98	93.86
14	3.09	17	0.08	20.27	53	6.67	63	0.30	94.16
15	3.18	13	0.06	20.33	54	6.90	11	0.05	94.21
16	3.22	10	0.05	20.38	55	7.19	13	0.06	94.27
17	3.31	14	0.07	20.44	56	7.30	60	0.28	94.55
18	3.44	99	0.46	20.90	57	7.37	33	0.15	94.70
19	3.48	29	0.14	21.04	58	7.46	31	0.14	94.85

20	3.54	17	0.08	21.12	59	7.72	23	0.11	94.95
21	3.59	9	0.04	21.16	60	7.90	9	0.04	94.99
22	3.62	9	0.04	21.20	61	8.03	30	0.14	95.13
23	3.68	129	0.60	21.80	62	8.27	6	0.03	95.16
24	3.78	7	0.03	21.83	63	8.37	7	0.03	95.19
25	3.85	2,966	13.81	35.64	64	8.64	16	0.07	95.27
26	3.93	54	0.25	35.89	65	8.93	62	0.29	95.55
27	4.01	1,177	5.48	41.37	66	9.05	12	0.06	95.61
28	4.11	25	0.12	41.49	67	9.11	8	0.04	95.65
29	4.15	8	0.04	41.53	68	9.18	38	0.18	95.83
30	4.22	50	0.23	41.76	69	9.45	8	0.04	95.86
31	4.27	2,482	11.55	53.31	70	9.72	62	0.29	96.15
32	4.36	34	0.16	53.47	71	9.86	14	0.07	96.22
33	4.43	12	0.05	53.52	72	10.01	11	0.05	96.27
34	4.51	810	3.77	57.29	73	10.12	64	0.30	96.56
35	4.65	39	0.18	57.48	74	10.30	716	3.33	99.90
36	4.71	37	0.17	57.65	75	10.46	7	0.03	99.93
37	4.78	56	0.26	57.91	76	10.75	6	0.03	99.96
38	4.96	73	0.34	58.25	77	10.98	8	0.04	100.00
39	5.04	7	0.03	58.29					

Table A.1.4 HPLC/DAD profile of *A. sydowii* FK1



Peak Number	Ret. Time	Area	% Area	Cumulative % Area	Peak Number	Ret. Time	Area	% Area	Cumulative % Area
1	0.53	840	7.25	7.25	29	4.71	11	0.09	70.09
2	0.80	112	0.97	8.22	30	4.78	83	0.71	70.80
3	0.86	200	1.73	9.96	31	4.94	27	0.23	71.03
4	1.03	66	0.57	10.53	32	5.05	9	0.08	71.11
5	1.30	31	0.27	10.80	33	5.15	123	1.06	72.17
6	1.56	212	1.83	12.63	34	5.19	94	0.81	72.98

7	2.23	35	0.31	12.93	35	5.35	15	0.13	73.11
8	2.46	24	0.21	13.14	36	5.47	10	0.09	73.20
9	2.69	46	0.40	13.54	37	5.60	31	0.26	73.47
10	2.95	368	3.17	16.71	38	5.71	20	0.17	73.64
11	3.03	7	0.06	16.77	39	5.78	39	0.34	73.97
12	3.08	655	5.66	22.43	40	5.87	94	0.81	74.78
13	3.18	7	0.06	22.49	41	5.97	14	0.12	74.90
14	3.28	371	3.20	25.70	42	6.16	107	0.92	75.82
15	3.36	71	0.61	26.31	43	6.32	2,405	20.76	96.58
16	3.44	986	8.51	34.82	44	6.43	9	0.07	96.66
17	3.51	12	0.11	34.92	45	6.62	9	0.08	96.73
18	3.62	272	2.35	37.27	46	6.90	10	0.09	96.82
19	3.73	271	2.34	39.61	47	7.30	54	0.46	97.28
20	3.86	707	6.11	45.72	48	7.90	8	0.07	97.36
21	3.95	7	0.06	45.77	49	8.07	13	0.11	97.47
22	4.01	479	4.13	49.91	50	9.05	18	0.16	97.62
23	4.16	51	0.44	50.35	51	9.18	9	0.07	97.70
24	4.27	2,149	18.56	68.91	52	9.65	8	0.07	97.76
25	4.33	13	0.12	69.03	53	9.72	22	0.19	97.95
26	4.45	6	0.05	69.07	54	10.12	14	0.12	98.08
27	4.52	62	0.53	69.61	55	10.31	217	1.87	99.95
28	4.65	45	0.39	70.00	56	10.98	6	0.05	100.00

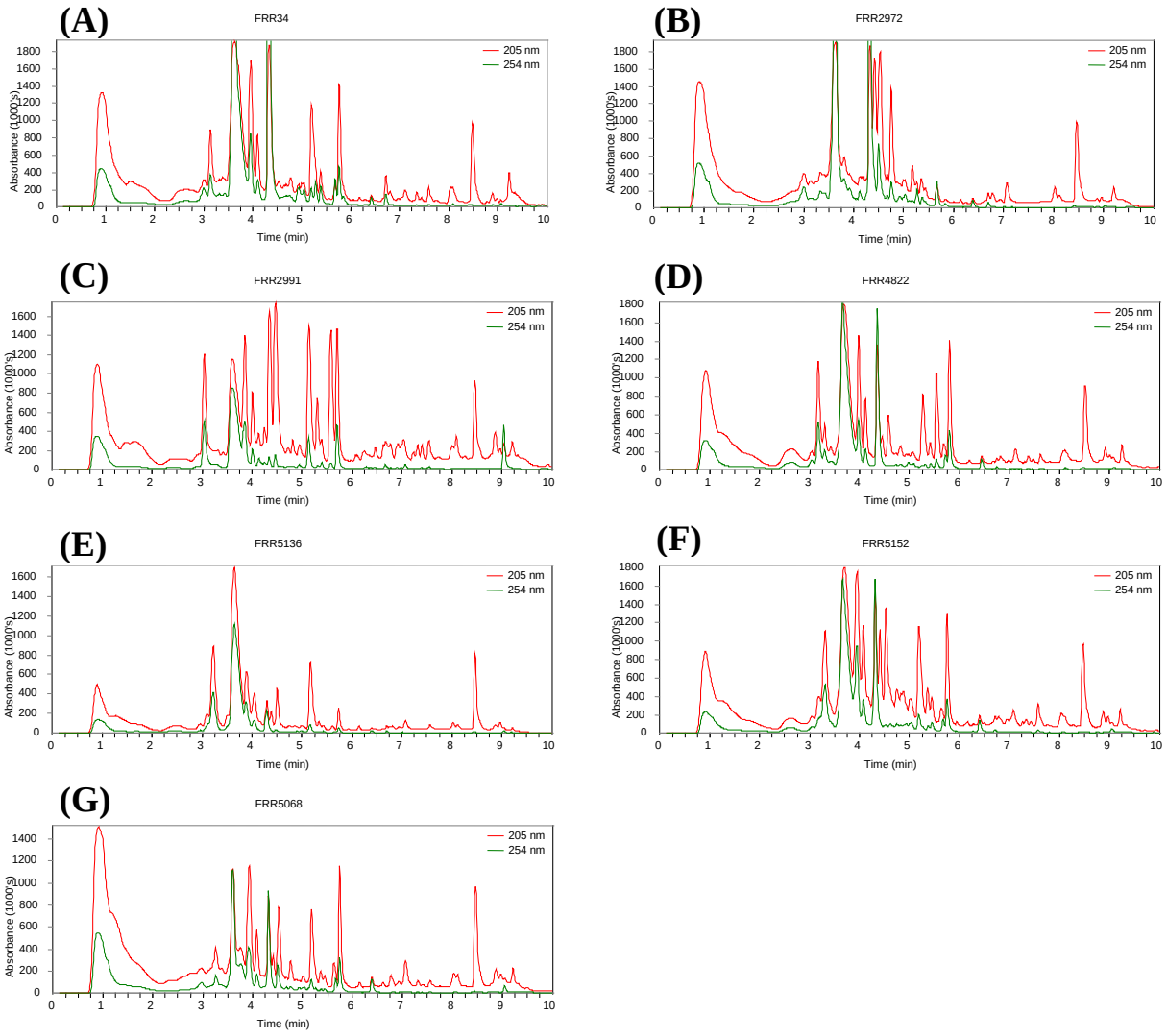


Figure A.1.1 HPLC profiles of *A. sydowii* investigated in the present study. The strains were cultured on rice for 7 days extracted with methanol (1 gm/mL) and analysed by gradient HPLC, (conditioned as described in body of the article). A. FRR34; B. FRR2972; C. FRR2991; D. FRR4822; E. FRR5136; F. FRR5152 and G. FRR5068.

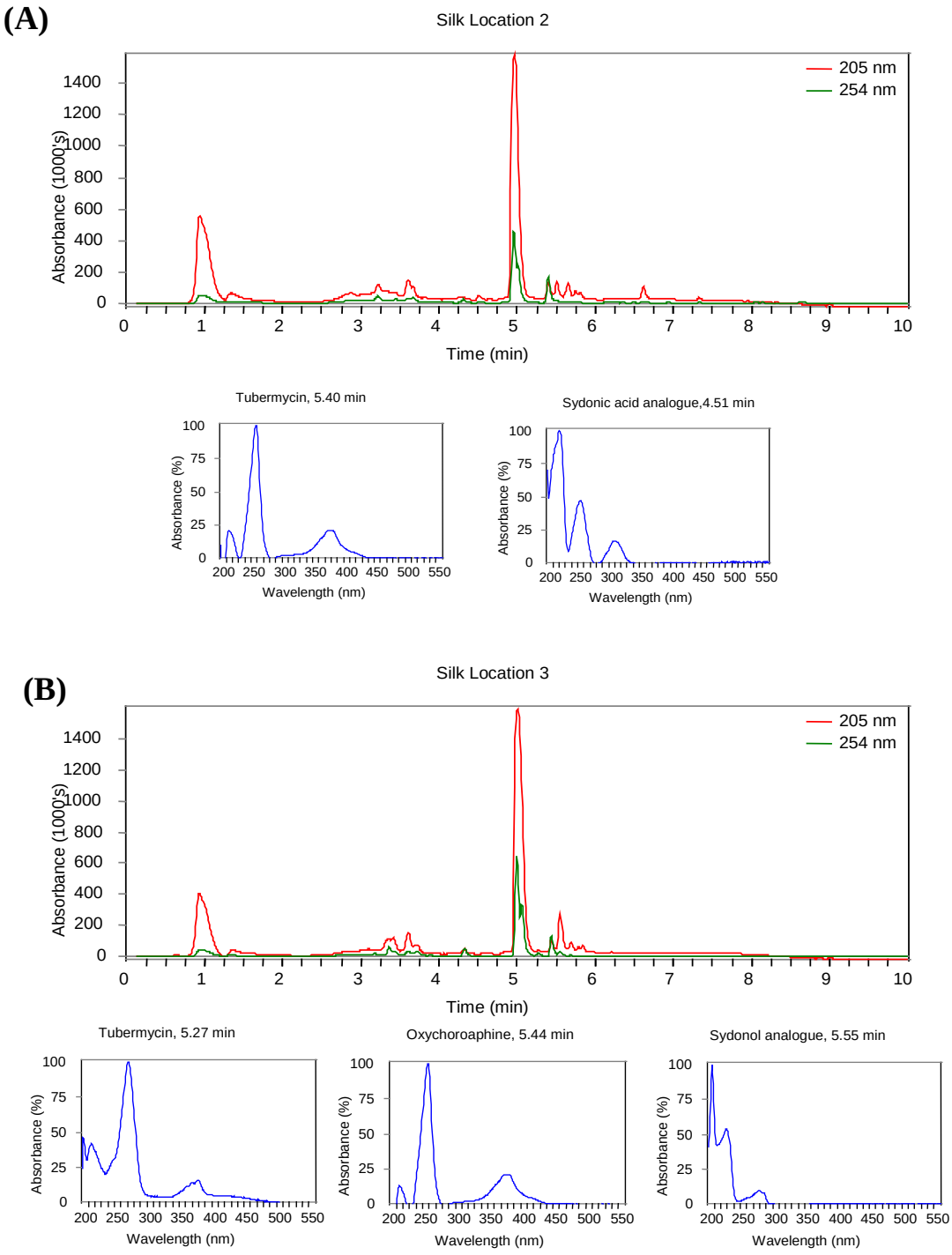


Figure A.1.2 HPLC profiles of extracted silks from A. location 2 and B. location 3, with the UV spectra of the microbial metabolites in the extract. (Note: The dominant peak eluting at 5 min, is an artefact of extraction of the silks with methanol)

A.2 Supplementary materials and methods for Chapter 5

A.2.1 Human neuroblastoma SH-SY5Y cell line culture

Human neuroblastoma SH-SY5Y was kindly provided by Ms Yilan Zhen and Dr. Kaylene Young (Menzies Institute for Medical Research, University of Tasmania, Australia). Cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM, D0819, Sigma-Aldrich) and 10% foetal bovine serum (FBS) (Bovogen Biologicals), and 100U/ml penicillin and 100mg/ml streptomycin solution in a humidified incubator (5% CO_2, 37°C). SH-SY5Y cells were routinely sub-cultured at a ratio of 1:30-1:50, and medium change-over occurred approximately every 5 d. Cell culture was sub-cultured <20 times upon receiving.

A.2.2 Phycotoxin standards

The details of mycotoxin and phycotoxin standards were previously described in Chapter 4, pp 56.

A.2.3 Differentiated neuroblastoma cytotoxicity bioassay

SH-SY5Y neuroblastoma cells were differentiated with sequential treatments of retinoic acid (RA, R2625, Sigma-Aldrich) with brain-derived neurotrophic factor (BDNF, B3795, Sigma-Aldrich), or staurosporine (STA, S6942, Sigma-Aldrich). Cells were differentiated with RA and BDNF, following the protocol developed by Encinas et al. (2002) with a few modifications. 100μL of cells suspension at 1.0×10^5 cells mL^{-1} were added to each well of 96 well plate, and allowed to attach for 24 h. Cells were further incubated with DMEM with 10 μM RA and 1% FBS for the first 6 days with media renewal at day 3. They were further incubated in DMEM with 25 ng/ml BDNF without FBS for the last 2 days before toxin exposure. SH-SY5Y cells were also differentiated with STA, following the protocols of Tieu, Zuo, & Yu (1999) with a few modifications. 100 μL of 5.0×10^4 cells mL^{-1} were added to each well of a 96 well plate. After 24 h, 50 μL of DMEM (supplemented with 10% FBS and antibiotics) containing STA were added to each well at the final STA concentration of 3×10^{-8} M. The cells were further incubated for 48h prior to toxin exposure.

As a comparison to STA differentiated cells, undifferentiated cells were also seeded at 5.0×10^4 cells mL^{-1} for 72h without any culture medium change-over. 24h toxin exposure and viability measurement were performed as previously described in subsection 5.2.3.

A.2.4 Data analysis

Statistical analyses were performed using R (R Core Team, version 3.4.3). Either student's t-test or One-way analysis of variance (ANOVA) was conducted to examine significant differences in cell viability among treatment. When a significant main effect was detected, Tukey's honestly significant difference (HSD) post hoc tests were conducted. Box–Cox transformation was applied to determine appropriate transformation to improve normality and homogeneity of variance. Data were express as mean ± standard deviation (SD) of four biological replicates (four wells). A significance level of 95% ($\alpha = 0.05$) was applied in this study.