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Dissertation

Effects, dynamics and management of okadaic acid in blue mussels, *Mytilus edulis*

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Abstract: Accumulation of algal toxins in mussels constitutes a serious threat to consumers and the industry. In Sweden, the annual occurrence of the diarrhetic shellfish toxin okadaic acid (OA) in blue mussels, *Mytilus edulis*, is the largest impediment to a further development of the mussel industry. OA is a potent protein phosphatase inhibitor and causes harmful effects at different levels within an organism. The effects of OA on mussels have previously not been investigated.

The inhibitory effects of OA on protein phosphatase (PP) and glycogen synthase (GS) activities in mussels and rainbow trout (*Oncorhynchus mykiss*) were studied. OA inhibited PP activity *in vitro* in both species. However, GS activity was inhibited by OA in rainbow trout but not in mussels. It was suggested that mussels may have mechanisms which prevent OA from binding to PP *in vivo*. Studies on the cytotoxic effects of OA in mussel blood cells showed that these cells are highly resistant to OA and it was hypothezised that this was due to p-glycoprotein activity (multixenobiotic resistance) in the cell membrane. However, the main site of p-glycoprotein activity was observed within lysosomal membranes. Preliminary results suggest that the lysosomes are involved in protecting the cells from the cytotoxic effects of OA.

Depuration of toxic mussels is a potential management option to increase the availability of marketable mussels. Various aspects regarding the physiological processes and the influence of environmental factors on depuration of OA were evaluated in manipulative experiments. Tests of the effects of food conditions showed that depuration rate was unaffected by feeding and digestive activities. In addition, the importance of lipid turnover for depuration of OA was evaluated. Although a positive correlation between lipid content and concentration of OA was observed in the field, depuration was not faster in mussels with reduced levels of lipids in the laboratory. Exposure to different temperatures and feeding conditions did not influence depuration rates. In a large-scale experiment, where toxic mussels were relocated to a fjord for depuration, a fast reduction of OA was obtained at the new location. However, these mussels accumulated DTX-1 which correlated to the presence of *Dinophysis acuta* in the fjord. A comparison between four different depuration experiments suggested that (1): depuration is equally effective in the lab as in the field (2): short-term manipulation of external conditions does not affect depuration rates (3): depuration is faster during summer compared to winter conditions. It was proposed that seasonal changes in the physiological status of the mussel regulate depuration of OA.

A field study comparing levels of OA among mussels, oysters (*Ostrea edulis*) and cockles (*Cerastoderma edule*) provided strong evidence for species-specific accumulation of this toxin and suggested that oysters and cockles can be marketed for human consumption during periods when harvest of mussels is banned.

Keywords: blue mussel, cockle, cytotoxic, depuration, diarrhetic shellfish poisoning, dinoflagellates, Dinophysis, environmental conditions, lipid, lysosome, management, multixenobiotic resistance, okadaic acid, oyster, p-glycoprotein, protein phosphatase

Till Mats och Emil

List of papers

This thesis is based on the following papers, referred to by their Roman numbers:

I. Svensson, S. Förlin, L. 1998. Effects of okadaic acid on protein phosphatase and glycogen synthase activities in the blue mussel, *Mytilus edulis*, and rainbow trout, Oncorhynchus mykiss. In: B. Reguera, J. Blanco, M.L. Fernandez and T. Wyatt (eds.) Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO. p 584-587

II. Svensson, S., Särngren, A., Förlin, L. (Accepted). Mussel blood cells resistant against the cytotoxic effects of okadaic acid do not express cell membrane p-glycoprotein activity (multixenobiotic resistance). Aquatic Toxicology.

III. Svensson, S. 2003. Depuration of Okadaic acid (Diarrhetic Shellfish Toxin) in mussels, *Mytilus edulis*, feeding on different quantities of nontoxic algae. Aquaculture 218 (1-4), 277-291.

IV. Svensson, S. Förlin, L. (Manuscript). Analysis of the relationship between lipid content and depuration of okadaic acid (diarrhetic shellfish toxin) in mussels, *Mytilus edulis*: results from a field study and a laboratory experiment.

V. Svensson, S., Norén, F. (Manuscript). Large-scale depuration of okadaic acid (Diarrhetic shellfish toxin) in farmed mussels, *Mytilus edulis*, by relocation to a fjord area on the Swedish west coast.

VI. Svensson, S., André, C., Rhenstam-Holm, A-S., Hansson, J. 2000. A case of consistent spatial differences in content of diarrhetic shellfish toxins (DST) among three bivalve species, *Mytilus edulis*, *Ostrea edulis* and *Cerastoderma edule*. J. Shellfish Res. 19(2), 1017-1020

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have already been published or are manuscripts at various stages (in press, accepted, submitted or in manuscript).

Contents

1. Introduction 7
1.1. General background and scope of this thesis 7
1.2. Harmful algal blooms 7
1.2.1. Paralytic Shellfish Poisoning (PSP) 8
1.2.2. Amnesic Shellfish Poisoning (ASP) 8
1.2.3. Neurotoxic Shellfish Poisoning (NSP) 9
1.2.4. Diarrhetic Shellfish Poisoning (DSP) 9
1.2.5. Algal toxins and shellfish 10
1.3. Okadaic acid 11
1.3.1. Mechanism of action 11
1.3.2. Secondary effects 11
1.3.3. Cytotoxicity 12
1.3.4. Multidrug resistance 12
1.4. The blue mussel 13
1.4.1. Ecology and life-history 13
1.4.2. Feeding and digestion 14
1.4.3. Aquaculture aspects 14
1.5. Bioaccumulation of lipophilic toxicants 16
1.5.1. Uptake mechanisms 16
1.5.2. Storage and metabolism 17
1.5.3. Depuration 18
1.6. Objective of this thesis 19
2. Detection methods 20
2.1. Mouse bioassay 20
2.2. High performance liquid chromatography (HPLC) 20
2.3. Protein phosphatase inhibition assay (PPIA) 21
3. Results and discussion 23
3.1. Intracellular responses and protective mechanisms 23
3.2. Depuration mechanisms and influence of environmental factors 27
3.3. Large-scale depuration 32
3.4. Depuration: summary and conclusions 34
3.5. Species-specific accumulation of DST 35
4. Concluding remarks 37
5. Acknowledgements 38
6. References 40

1. Introduction

1.1. General background and scope of this thesis

In 1997, an interdisciplinary project (SUCOZOMA) was initiated and financed by the Swedish Foundation for Environmental Strategic Research (MISTRA). This project deals with various aspects of coastal resource management including fisheries and water quality issues. A part of the project has been to evaluate the use of mussel farms as biofilters to absorb discharge of nutrients and as a complement to conventional sewage treatments in a eutrophicated fjord system on the Swedish west coast (Haamer, 1996; Haamer *et al.*, 1999). The general increase in discharge of nutrients from land to sea is currently seen as one of the most serious threats to coastal environments. Excess of nutrients accelerate blooms of phytoplankton and consequently the potential for eutrophication effects such as oxygen depletion which are detrimental to organisms in sediments and water columns. The idea to use mussels as biofilters for nutrients is based on the high capacity of mussels to capture phytoplankton before they sink to the bottoms and in this way transform nutrients into mussel meat. The mussels are intended to be used for human consumption. However, some phytoplankton species produce toxic compounds which accumulate in mussels and may cause various seafood poisoning syndromes in humans. In Sweden, the accumulation of diarrhetic shellfish toxins (DST) in blue mussels, *Mytilus edulis*, is by far the largest impediment to a sustainable mussel industry today and there is a need for management plans to reduce the negative effects of DST (Kollberg, 1999). Depuration of toxic mussels has been proposed as a potential option for mussel farmers. This would allow a continuous supply of mussels. In order to develop practical and cost-effective methods for depuration, extensive knowledge about variations in rates of depuration of DST in mussels during different environmental conditions are needed. In this context, it is important to identify of the physiological processes by which mussels accumulate and eliminate these compounds. The physiological response in mussels during toxic events was poorly understood at the start of these studies. The aim of this thesis is to investigate some issues concerning the effects and dynamics of DST in the blue mussels.

1.2. Harmful algal blooms

Blooms of marine microalgae constitute the basis for all living organisms in the sea and most of the time, these blooms have no detrimental effect on the environment. However, some species occasionally occur in such large numbers, or produce toxic compounds, that eventually cause harm to other organisms. Harmful algal blooms (HAB) have been defined as events where the concentration of algae reaches levels which can kill fish or shellfish directly, or indirectly by causing oxygen depletion, or cause accumulation of toxins in marine organisms which eventually harm other organisms who eat the toxic species (Hallegraeff, 1993; Andersen, 1996). Most of the toxic microalgae in the marine environment belong to the dinoflagellate group (Landsberg, 2002). Although HAB are completely natural

phenomena, there is an observed increase in frequency and numbers of algal species producing toxins in the past two decades. This has been attributed to antropogenic activities such as eutrophication of coastal areas (Smayda, 1990; Hallegraeff, 1993; Van Dolah, 2000b).

Algal toxins are so-called secondary metabolites which means that they have no known key role in the basic functioning of the producing algal cell in which they occur. However, there are several theories regarding the functional significance of secondary metabolites in the algae, including their putative function as chemical defence agents, hormonal and ion channel regulators as well as regulators of cell division (Windust *et al.*, 1996; Aguilera *et al.*, 1997; Wright & Cembella, 1998).

Humans experience the effects of HAB when consuming toxic shellfish or fish causing various seafood poisoning syndromes. Contamination of shellfish, predominantly bivalve molluscs, by algal toxins is one of the most serious problems for the aquaculture and fisheries industries world-wide today, causing major economical losses and bad publicity for seafood as a food resource (Shumway, 1990; Bricelj & Shumway, 1998; Vieites & Leira Sanmartin, 2000). Fortunately, most countries where bivalves are exploited for food production have ongoing monitoring programs to detect toxins in both algae and shellfish. These programs have markedly reduced the incidences of human intoxication in recent years (Andersen, 1996). Nevertheless, the negative effects of HAB have created a great demand for management and mitigation strategies to reduce the occurrence of toxic shellfish. The most obvious way is perhaps to put efforts into finding locations for aquaculture where prevalence of toxic algal species are low, although there is no 100% guarantee that a certain area will be unaffected by future HAB. Early warning systems based on monitoring of potential toxic species can be effective and create opportunities for farmers to take measures before the mussels become intoxicated. A different strategy is to depurate live shellfish containing toxins and thereby achieve a more regular supply throughout the year (**paper III, IV and V**). However, there are no universal methods available for depuration, potential methods have to be tailor-made for each bivalve species and toxin.

A brief summary of the common syndromes associated with consumption of toxic shellfish follows below. Diarrhetic shellfish poisoning (DSP) and the causative toxic compounds will be described in more detail due to their relevance for the topic of this thesis.

1.2.1. Paralytic Shellfish Poisoning (PSP)

This is the most serious toxin-related seafood disease because of its lethal potential and worldwide distribution. Approximately 2000 cases are reported per year with an average mortality rate of 15% (see reviews by Van Dolah, 2000a; Bricelj & Shumway, 1998). Clinical symptoms of PSP include initial numbness of the lips and face, spreading to the extremities with subsequent loss of motor control and, in extreme cases, respiratory paralysis and death. PSP is caused by a group of water-soluble compounds collectively called saxitoxins which affect the central nervous system of mammals. They act by binding to voltage-dependent sodium channels, thus inhibiting channel conductance and thereby blocking neuronal activity in the peripheral nervous system. The saxitoxins are produced by dinoflagellates of the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium*.

1.2.2. Amnesic Shellfish Poisoning (ASP)

Similar to PSP, ASP is caused by a water-soluble neurotoxic agent known as domoic acid (Wright *et al.*, 1989). This is the only known toxin produced by diatoms

(*Pseudonitzschia* spp.) and these have been observed in temperate waters globally, however, no human outbreaks have occurred since the discovery in Canada in 1987 (Van Dolah, 2000b). Domoic acid is a tricarboxylic amino acid and acts as an analogue of glutamate, which is a neurotransmitter. Persistent activation of the glutamate receptor by domoic acid results in increased levels of intracellular Ca^{2+} with subsequent lesions in some areas of the brain (Peng *et al.*, 1994). Symptoms of ASP include gastrointestinal effects as well as neurological effects such as dizziness, lethargy, seizures and permanent loss of short-term memory.

1.2.3. Neurotoxic Shellfish Poisoning (NSP)

This type of seafood poisoning is caused by brevetoxins, a group of polycyclic ether compounds with potent neurotoxicity and hemolytic properties. The predominant causative species is the dinoflagellate *Karenia brevis* (= *Gymnodinium breve*) and NSP incidents are so far restricted to the Gulf of Mexico, the east coast of Florida and New Zealand (Landsberg, 2002). Brevetoxins bind to voltage-dependent sodium channels, inhibiting channel inactivation which result in prolonged channel opening (Poli *et al.*, 1986). Symptoms of NSP are nausea, numbness of mouth and face, loss of motor control and muscle pain. Severe cases may result in unconsciousness. An additional route of human exposure to brevetoxins is through respiration of aerosolized toxins causing symptoms of irritation and burning of the respiratory tract.

1.2.4. Diarrhetic Shellfish Poisoning (DSP)

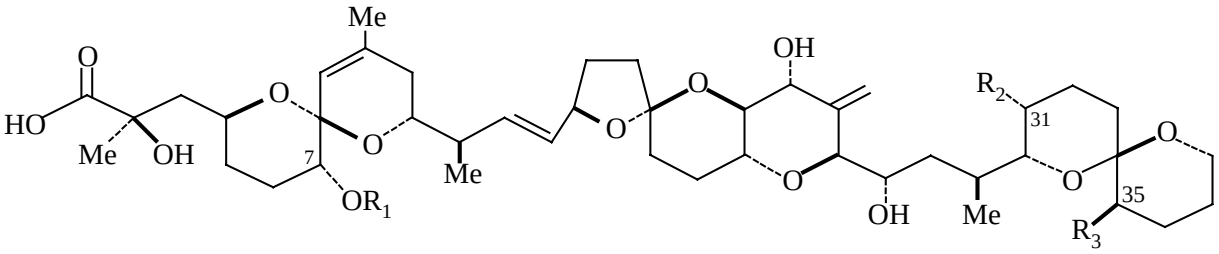
DSP was first described in Japan in 1977 when an outbreak of gastroenteritis, not caused by pathogenic bacteria, occurred after consumption of mussels and scallops (Yasumoto *et al.*, 1978). The major symptoms, which appeared within 30 minutes to 7 hours after consumption, were diarrhoea, nausea, abdominal pain and vomiting. Patients recovered within 3 days. DSP incidents were later reported from Europe and this syndrome is now recognised in all parts of the world (Kat, 1983; Krogh *et al.*, 1985; Underdal *et al.*, 1985; Aune & Yndestad, 1993; Van Dolah, 2000b). DSP is caused by the lipophilic polyether compounds okadaic acid (OA) and the OA derivatives Dinophysistoxin-1 (DTX-1), DTX-2 and DTX-3 (fig. 1), all of which accumulate predominantly in the digestive organs of filter-feeding organisms (Yasumoto *et al.*, 1978; Murata *et al.*, 1982; Yasumoto *et al.*, 1985; Kumagai, 1986; Edebo, 1988). OA was first isolated from a marine sponge, *Halicondria okadai* (Tachibana *et al.*, 1981), but the causative DSP species were identified as dinoflagellates of the *Dinophysis* and *Prorocentrum* genera (Yasumoto *et al.*, 1980; Murakami *et al.*, 1982; Lawrence *et al.*, 1998). A summary of species known to produce DSP toxins (DST) is published by Landsberg (2002). DTX-3 is a common reference name for a group of homologues in which the 7-OH position of OA, DTX-1 or DTX-2 is esterified with different kinds of fatty acids, the most common being palmitic acid (fig. 1). Because the DTX-3 compounds was not detected in the causative algal species, they were formerly believed to be exclusive biotransformation products in the bivalve tissue (Yasumoto *et al.*, 1985; Lee *et al.*, 1989). However, DTX-3 was recently detected in similar proportions in both *Dinophysis acuta* and greenshell mussels *Perna canaliculus* from New Zealand, indicating that DTX-3 can also be assimilated by the mussel through feeding (MacKenzie *et al.*, 2002). OA was initially identified as the dominating DSP toxin in European mussels but other derivatives are now regularly found all over this continent (Kumagai, 1986; Marr *et al.*, 1992; Carmody *et al.*, 1996; Vale & Sampayo, 2002b). In Sweden, OA is the most common DST detected in blue mussels, *M.*

edulis. The structurally unrelated pectenotoxins (PTX) and yessotoxins (YTX) have been grouped within the DSP complex since they are extracted from mussels using the same initial procedures as OA and DTX. However, PTX and YTX are not protein phosphatase inhibitors and are not diarrhogenic when orally administered. They should therefore not be regarded as DSP toxins (Hamano *et al*, 1986; Aune, 1997; Daranas *et al.*, 2001; Aune *et al.*, 2002). Rather, PTX and YTX are potent hepatotoxic compounds and it has tentatively been proposed that they should form a new group of toxins called hepatotoxic shellfish poisoning (HSP) toxins (Daranas *et al.*, 2001). The recently discovered azaspiracids (AZA) in mussels from Ireland, UK and Norway produce a gastrointestinal intoxication in humans with similar symptoms as DSP (James *et al.*, 2002). The AZA have unique structural features and toxicological effects in mice and this food poisoning syndrome has been named azapiracid poisoning (AZP).

1.2.5. Algal toxins and shellfish

Algal toxins which affect humans may also cause lethal or sublethal effects in vector organisms such as the bivalve molluscs (Bricelj & Shumway, 1998; Landsberg, 2002). Reported cases of mass mortalities of bivalves due to HAB obviously result in great economic loss and pose an additional problem for the shellfish industry (Underdal *et al.*, 1989; Shumway, 1990). The effects of different toxins vary greatly among different species. Large interspecific differences in both accumulation and elimination of specific toxins are documented. This phenomena can be exploited in the selection of candidate species for monitoring purposes or aquaculture activities (Bricelj & Shumway, 1998; Landsberg, 2002; **paper VI**). In some cases, species-specific patterns of accumulation of toxins have been correlated to sensitivity to the toxin. The most documented examples concern the response on nerve cells to paralytic shellfish toxins (Bricelj & Shumway, 1998). In this context, *Mytilus* sp. is generally regarded as a species insensitive to most toxins. These species therefore readily feed and accumulate high toxin levels, but also detoxify more rapidly compared to other species.

So far, there is no documentation from the



	R_1	R_2	R_3
Okadaic acid (OA)	H	CH_3	H
Dinophysistoxin-1 (DTX-1)	H	CH_3	CH_3
Dinophysistoxin-2 (DTX-2)	H	H	CH_3
Dinophysistoxin-3 (DTX-3)	acyl	CH_3	CH_3

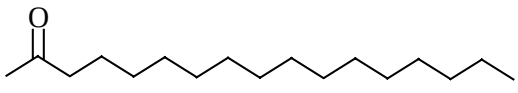
typical acyl group  (C16:0)

Fig. 1. Chemical structure of the causative agents of diarrhetic shellfish poisoning (DSP), okadaic acid and the dinophysistoxins.

field or laboratory which suggest that the survival of mussels are negatively affected by the DSP toxins (Landsberg, 2002). In Sweden, blue mussels contain high concentrations of OA (occasionally above 1000 µg*kg^{-1} mussel meat), during extensive periods yearly (Lindegarth, 1997; Karlsson & Rehnstam-Holm, 2002). However, no increased mortalities have been reported in correlation to these events. Likewise, no mortality was observed in a laboratory experiment where *M. edulis* were fed a unialgal diet of the OA-producer *Prorocentrum lima* for one month (Pillet *et al.*, 1995). Although the DST do not appear to cause acute effects in mussels, the possibility remains that chronic exposure to these compounds may negatively affect the fitness of the organisms (Landsberg, 1996). It also seems likely that organisms frequently exposed to DST-producing dinoflagellates, such as the blue mussel, have evolved protective mechanisms (**paper I, II**).

1.3. Okadaic acid

1.3.1. Mechanism of action

The biological activity and underlying mechanism of toxicity for OA has been widely studied (see review by Vieytes *et al.*, 2000). OA is a potent inhibitor of serine/threonine protein phosphatase activity, predominantly protein phosphatase 1 (PP1) and PP2A (Bialojan & Takai, 1988), which are enzymes responsible for dephosphorylation of proteins. Reversible, covalent phosphorylation is a universal cellular mechanism which regulates protein activities (Cohen & Cohen, 1989; Cohen, 1989; Cohen, 1994; Wera & Hemmings, 1995). The phosphorylation state and activity of a protein is a dynamic process controlled both by protein kinases, responsible for phosphorylation, and protein phosphatases which dephosphorylate the same proteins. OA disturbs this control mechanism by binding to the protein phosphatases, thus inhibiting dephosphorylation. This results in hyperphosphorylation of a wide range of enzymes with subsequent secondary effects on intracellular signalling pathways (Haystead *et al.*, 1989). OA interacts with the phosphatases by binding to the catalytic subunit with a 200-fold higher affinity for PP2A in cell-free extracts (IC_{50} 0.07 nM) than PP1 (3.4 nM). (Takai *et al.*, 1992). The maximum inhibitory concentration of OA on PP1 and PP2A in intact cells is, however, much higher (1 µM) which is similar to the *in vivo* concentration of PP1 and PP2A (Cohen, 1989). Structure-affinity studies have suggested that the carboxyl group, the hydroxyl group at C-24 and the particular spatial conformation of the OA molecule are all essential for its inhibitory activity on the phosphatases (fig. 1) (Holmes *et al.*, 1990; Nishiwaki *et al.*, 1990). The difference in specificity for PP1 and PP2A has resulted in OA being used as a tool for studying cellular processes regulated by reversible phosphorylation and to distinguish between different classes of PP (Haystead *et al.*, 1989; Cohen *et al.*, 1990). This approach which was used in **paper I** to study protein phosphatase activities in mussel and rainbow trout (*Oncorhynchus mykiss*) tissue. The inhibitory properties of OA on protein phosphatases are also used for quantification of this toxin in shellfish and plankton extracts and several bioassays using purified PP2A have been developed (see *2.3* and **paper IV**, **V** and **VI**).

1.3.2. Secondary effects

The general effects of OA on protein phosphorylation affect many cellular processes. For example, hyper-phosphorylation of the rate-limiting enzymes of glycogen metabolism, glycogen phosphorylase and glycogen synthase (Roach, 1981), have been shown to result in increased breakdown of glycogen stores as well as inhibiting glycogen synthesis *de novo*.

(Haystead *et al.*, 1989; Pugazhenthi *et al.*, 1993). Glycogen synthase (GS) was used as a model enzyme in **paper I** to investigate the effects of OA in blue mussels compared with rainbow trout. The dephosphorylated form of GS is active *in vivo*, thus the inhibition of protein phosphatases by OA was predicted to similarly inhibit glycogen synthase activity.

Other effects associated with OA include increased production of oxygen radicals (Schmidt *et al.*, 1995) and induction of apoptosis (Boe *et al.*, 1991; Kawamura *et al.*, 1996; Ritz *et al.*, 1997; Fernández-Sánchez *et al.*, 1998). OA is also a potent tumour promoter in such a way that hyperphosphorylation activates pathways which lead to the expression of several cell proliferation genes (Suganuma *et al.*, 1988; Fujiki & Suganuma, 1993; Sueoka & Fujiki, 1998). The diarrhogenic effects of OA are likely to be the result of increased phosphorylation of enzymes regulating sodium secretion and cytoskeletal or junctional elements in the intestinal cells (Cohen *et al.*, 1990; Aune & Yndestad, 1993). Increased intestinal paracellular permeability by OA has also been shown to impair water balance and increase loss of fluids (Tripuraneni *et al.*, 1997). 40 µg OA is sufficient to induce diarrhoea in adults (Hamano *et al.*, 1986).

1.3.3. Cytotoxicity

As might be suspected, low concentrations of OA is highly cytotoxic in a vast range of cell culture systems, including mammalian and fish cell lines (Tohda *et al.*, 1994; Tubaro *et al.*, 1996b; Laidley *et al.*, 1997; Ritz *et al.*, 1997; Fladmark *et al.*, 1998). Prolonged inhibition of PP1 and PP2A triggers the signalling cascade for apoptotic cell death, which seems to be the underlying mechanism for the cytotoxicity of OA (Ritz *et al.*, 1997). LC_{50} values reported for OA range from 1.5 nM in human epidermoid carcinoma cells (KB) cells (Tubaro *et al.*, 1996b), 13 nM in Chinese hamster ovary cells (CHO-K1; Tohda *et al.*, 1994), 20 nM in salmon hepatocytes (Fladmark *et al.*, 1998), 30 nM in rat pituitary GH_3 cells (Ritz *et al.*, 1997) and 31 nM in mouse neuroblastoma cells (N1E-115; Laidley *et al.*, 1997). Various bioassays have been developed in order to quantify OA in shellfish extracts where the cytotoxic properties of OA are exploited (Aune *et al.*, 1991; Amzil *et al.*, 1992; Tubaro *et al.*, 1996b; Fladmark *et al.*, 1998). These methods are generally sensitive for extracts where OA is the only toxic compound but they become unspecific if other toxins are present in the tissue rendering them unsuitable for monitoring purposes. There are no previous studies investigating the cytotoxic effects of OA in mussels, an organism which is naturally exposed to this toxic compound. In **paper II**, the cytotoxic effects of OA in mussels were studied, using blood cells as a model cell system.

1.3.4. Multidrug resistance

Some cell lines which have been exposed to non-toxic concentrations of OA for some time have been shown to develop resistance against the cytotoxic effects of this toxin. This resistance has been attributed to multidrug resistance (MDR) mechanisms (Tohda *et al.*, 1994; Wang *et al.*, 1995; Ritz *et al.*, 1997). MDR is a term used in cancer research to describe mammalian tumour cells which have become resistant to cytotoxic drugs (see reviews by Endicott & Ling, 1989; Ambudkar *et al.*, 1999). The corresponding mechanism in non-mammalians has been termed multixenobiotic resistance (MXR; Bard, 2000). The common feature of MDR and MXR is the overexpression of a plasma membrane phosphoglycoprotein (p-gp). This transmembrane protein functions as an ATP-dependent, drug efflux pump. It reduces the intracellular levels of various, structurally unrelated, lipophilic compounds against a concentration gradient (fig. 5A). Thus, the

reduced intracellular concentrations of the toxic agents, by the action of p-gp, positively affect the survival of the cells. The P-gp system is not only used for transport of foreign compounds but also for cellular excretion of endogenous substances (Higgins, 1992; Ambudkar *et al.*, 1999). Cell lines expressing MDR phenotype have been found to be resistant to the cytotoxic effects of OA (Chambers *et al.*, 1993). It has been suggested that OA is a substrate for p-gp since the observed resistance can be reversed by the addition of verapamil, a competitive inhibitor of p-gp activity (Yusa & Tsuruo, 1989; Tohda *et al.*, 1994; Ritz *et al.*, 1997).

Mussel blood cells were found to be highly resistant against the cytotoxic effects of OA and the hypothesis that this was due to p-gp activity in blood cell membranes was tested (**paper II**). P-gp expression and activity have been confirmed previously in various tissues of *Mytilus* sp. including the blood cells (Kurelec & Pivcevic', 1991; Minier *et al.*, 1993; Cornwall *et al.*, 1995; Kurelec, 1995; Minier & Moore, 1996).

1.4. The blue mussel

1.4.1. Ecology and life-history

The blue mussel is distributed throughout the temperate waters of both the northern and southern hemisphere (Seed & Suchanek, 1992). It is a dominating species on intertidal, wave-exposed hard substrates although dense masses of mussels are also common in subtidal, shallow areas. Blue mussels tolerate a wide range of environmental conditions and show a remarkable plasticity of physiological response to changes in the surroundings, thus the limiting distribution factors are biological (i.e. predation and competition) rather than physical or chemical. Being a common and dominating species, the ecological significance of the blue mussel is high. For example, filtration activities of the mussel population in Öresund (Baltic Sea) influence the entire water column, changing the abundance and composition of the phytoplankton community and hence the flux of nutrients (Haamer & Rodhe, 2000). Mussel beds also represent a structural habitat for a diverse range of organisms, creating there own local community "ecosystem" (Seed & Suchanek, 1992). Blue mussels are widely used as indicators of environmental pollution (the "Mussel Watch" concept). There are several attributes which make them ideal for biomonitoring purposes (world-wide distribution, sedentary organisms, bioconcentrators of chemical pollutants etc.; Widdows & Donkin, 1992).

Mytilus sp. have separate sexes and both male and female mussels mature after approximately one year. Eggs and sperms are released directly into the open water where external fertilization takes place. A planktotrophic larval stage (veliger stage) develops within 48 hours and the free-living larval stage typically persists for three to four weeks. The larvae is then ready to find a suitable substrate for settlement and develops into an adult, sessile life-stage.

In higher latitudes, mussels show a pronounced annual cycle of reproduction (Seed & Suchanek, 1992). The various reproductive stages can be assessed by measuring the gonad development using histological methods (Seed, 1969). The reproductive cycle of the Skagerrak blue mussel populations have been documented by Loo & Rosenberg (1983) and Svärdh (2003). In general, development of the gonads takes place during winter and early spring. In April, most of the gonads are ripe and a period of spawning follows which may continue into the early summer months. In August, the gonads are spent and the mussels enter a reproductive quiescent phase until the end of the year when gonads start to redevelop again. The reproductive stages are strongly coupled to glycogen content in the mantle tissue (Svärdh, 2003) where glycogen is used as fuel for the gametogenesis. A complex interaction

between external factors (e.g. food, temperature, salinity) and endogenous systems (e.g. nutrient reserves, hormonal cycles, genotype) in determining the initiation and duration of gametogenesis seem to exist (De Zwaan & Mathieu, 1992).

1.4.2. Feeding and digestion

Mussels feed on a variety of seston particles ranging from approximately 1 to 200 µm in size. The diet include bacteria, phytoplankton, microzooplankton and detritus (see review by Morton, 1983; Jörgensen, 1991; Hawkins & Bayne, 1992 and also Hernroth *et al.*, 2000). Coordinated ciliary movements on the gills are used to actively pump water through the mantle cavity. During this process, food particles are captured by mucociliary mechanisms on the gills and labial palps. The particles are transported along ciliated grooves to the labial palps for ingestion through the mouth or rejection as pseudofaces. At low densities, all food particles are ingested but at seston concentrations above a threshold, corresponding to the maximum digestive capacity of the mussel, the surplus filtrated material is bound with mucus to form pseudofaeces and is rejected prior to ingestion. Mussels also have the ability to preferentially select and ingest specific particles from a mixture of food (Newell *et al.*, 1989; Ward *et al.*, 1998). Food destined for ingestion enter the stomach where extracellular digestion takes place (Reid, 1965). Particles are mechanically disintegrated by the crystalline style structure which also releases enzymes for chemical degradation. Food material is then transported through the sorting caecum for further intracellular digestion in the digestive diverticula or directly transported to the gut system, which also has an absorptive and digestive role (Morton, 1983). In the digestive diverticula, food particles are absorbed by endocytosis or pinocytosis into the digestive cells for intracellular degradation where the lysosomal system plays a significant role (Owen, 1972). The lysosomes contain hydrolytic enzymes which further degrade the food particles. Rest-products contained in residual bodies are expelled from the cells by exocytosis and excreted via the intestine. The regulation of the digestive activities is coupled to the amounts of food ingested where the ingested amount is positively correlated to stomach and gut passage time. This affects the efficiency of degradation and absorption of nutrients from the food. Mussels show an endogenous rhythmicity which coordinates the mechanisms of feeding and digestion (Langton, 1977; Hawkins *et al.*, 1983). The processes involved in feeding and digestion also show marked seasonal changes in rates and this is connected to the reproductive cycle and the physiological status of the mussels (Hawkins & Bayne, 1984; Hawkins & Bayne, 1985; Hawkins & Bayne, 1992). As evident from above, mussels have a wide variety of physiological response to the food resource, enabling them to regulate food acquisition according to their needs (Hawkins & Bayne, 1992).

1.4.3. Aquaculture aspects

Aquaculture and fisheries for mussels (fig. 2) are growing in importance as fish catches decrease in all parts of the world (Vieites & Leira Sanmartin, 2000). Mussels are an ideal candidate for aquaculture purposes, compared to fish and other bivalve molluscs (Hickman, 1992). (1). No rearing of larvae is necessary where mussel cultivation depends on the natural supply of larvae. (2). Mussels attach to surfaces naturally with byssal threads, a major advantage compared to other bivalve species and greatly affects farming practices and technological development. (3). No food has to be supplied, mussels feed on the natural phytoplankton community and have a rapid growth rate. (4). Mussels, even in very high densities, are generally resistant to

Fig. 2. Cultured mussels attached to strips in a long-line mussel farm (Photo: Lars-Ove Loo).

diseases and mass mortalities are rare. The direct conversion of nutrients (through phytoplankton) by mussels, generating a high-quality food product is perhaps the most appealing characteristic of mussels in aquaculture.

Mussel production comes mainly from culture (80%) with only 20% from catches of wild mussels. World production has been around one million tons since 1990 (FAO statistics) but this number is predicted to increase during the next decade since "new" countries have started to produce mussels. Two large mussel-producing areas can be distinguished; China and other Asian countries (46%) and Europe (41%; Vieites & Leira Sanmartin, 2000). In Europe, mussel production is dominated by Italy (116 000 tons per year), Spain (92 000), France (75 000) and the Netherlands (79 000; FAO statistics, 1995). In Denmark, large quantities (>100 000 tons per year) of wild mussels are harvested in Limfjorden and mainly used in the canning industry. In this context, Swedish mussel production is a small-scale industry with an average yearly production of 2 000 tons (Kollberg, 1999).

Various methods to culture mussels are used. These can be divided into two categories; on-bottom (two-dimensional) and off-bottom (three-dimensional). The on-bottom method is based on transferring naturally occurring mussels (10-30 mm) to culture plots where they are spread in lower densities and growth is stimulated. This method dominates in the Netherlands where a highly sophisticated and mechanized industry using this cultivation method has developed (Hickman, 1992). Off-bottom methods use either poles sticking up from the bottoms or ropes hanging down from rafts or anchored buoys in the water column where mussels attach and grow. The off-bottom approach has several advantages compared to on-bottom cultivation; growth rate is twice as high due to more food being available in the water column; better protection against predation by seastars; better areal usage since the off-bottom methods are three-dimensional. Mussel rafts are common in Spain whereas in Sweden, the long-line system is used (fig. 3; Haamer, 1995a). The average long-line mussel unit in Sweden produces 150-200 tons of mussels in 18-24 months.

As mentioned previously, the occurrence of algal toxins is the most serious threat to the culture and processing of mussels today (Shumway, 1990; Vieites & Leira Sanmartin, 2000). As an example, an outbreak of DSP in Sweden in 1984 (Krogh *et al.*, 1985; Underdal *et al.*, 1985) had devastating effects on the expanding mussel culture and the industry. It took several years to recover. The establishment of a monitoring program for DST in 1985 has reduced the incidences of DSP in humans but mussel growing areas may be closed for harvest for up to six months every year due to elevated levels of DST (Edebo, 1988; Haamer, 1990b;

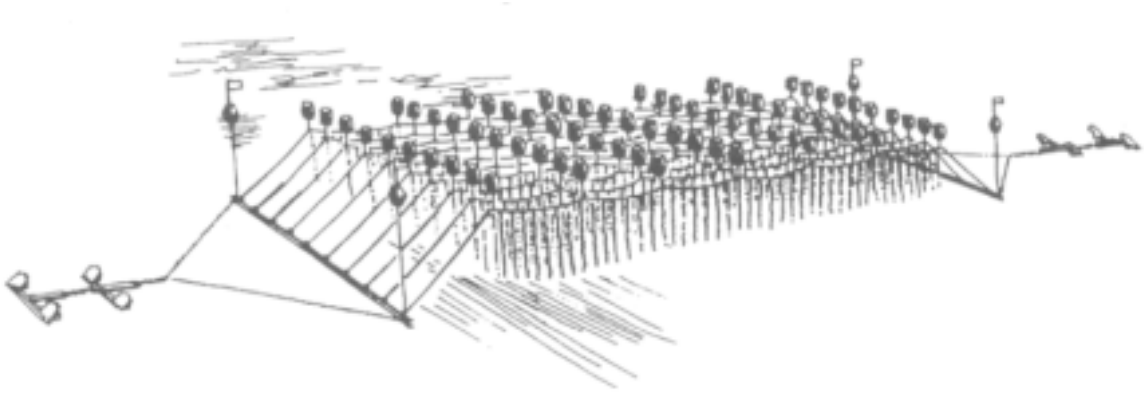


Fig. 3. The Swedish long-line system with the dimensions 10 x 200 m. The long-lines can carry 20 000 m of farming strips with a production capacity of about 200 tons in two years. From Haamer (1995a).

Lindegarth, 1997). Although a re-establishment of mussel farming activities is occurring today, the presence of DST in mussels is still a major problem for this industry in Sweden (Kollberg, 1999).

1.5. Bioaccumulation of lipophilic toxicants

1.5.1. Uptake mechanisms

Blue mussels, being suspension-feeders and pumping large volumes of water, accumulate and concentrate a wide variety of lipophilic organic contaminants and algal toxins (referred to as toxicants in section *1.5*) in their tissue (Moore *et al.*, 1989; Landsberg, 2002). In general, bioaccumulation is a function of the uptake rate of the toxicant and the elimination rate of the same (Spacie & Hamelink, 1985; Phillips, 1993). The degree to which the toxicants are bioaccumulated depends upon both the physicochemical properties of the compound (solubility, hydrophobicity, free versus particulate-bound etc.) and biotic factors such as the pumping activity, biochemical composition, reproductive condition, metabolic activities and elimination in the mussels (Widdows & Donkin, 1992). Both the abiotic and biotic factors are in turn affected by environmental variables such as temperature and salinity which influence the dynamic processes concerned with uptake and depuration of toxic agents.

The major uptake routes of organic pollutants in mussels are via passive diffusion across membranes of the gills or by filter-feeding on the particulate-bound fraction in the water column and assimilation in the digestive system (Spacie & Hamelink, 1985; Livingstone & Pipe, 1992). Uptake of lipophilic algal toxins such as OA is assumed to occur exclusively by feeding on the toxin-producing algae and hence the resulting assimilation of the toxins into the tissue takes place within the digestive system (Landsberg, 2002). No further distribution of OA to other organs has been observed in mussels (Yasumoto *et al.*, 1978; Edebo *et al.*, 1988). Therefore, factors determining the rate of uptake of this toxin should be connected to the feeding physiology and availability of the toxin-producing dinoflagellates in the plankton community. In this context, both the absolute and relative abundance of toxic algae, together with total amount of food available for the mussels, are important variables affecting general filtration and ingestion rates (Sampayo *et al.*, 1990; Hawkins & Bayne, 1992; Morono *et al.*, 1998; Blanco *et al.*, 1999). Additionally, the OA content per cell in the causative species (*Dinophysis* sp.) may vary considerably both temporally and spatially and this is another factor affecting the rate of uptake of this toxin (**paper V** and Lee *et al.*, 1989; Andersen *et al.*, 1996; Aune *et al.*, 1996; Dahl & Johannessen, 2001; Fernández *et al.*, 2002; Marcaillou *et al.*, 2002). The rate of

assimilation of OA in the digestive system is likely to be affected by the total amounts of ingested food since this affects the gut passage time and probability for assimilation through the gut wall (Hawkins & Bayne, 1992; Blanco *et al.*, 1999). The abundance of food is also important in determining the bioaccumulation of organic contaminants in mussels (Björk & Gilek, 1997; Magnusson *et al.*, 2000). Apart from all aspects regarding the food resource, any other factor which affects the feeding physiology of mussels are likely to influence the uptake of toxicants including OA in mussels.

1.5.2. Storage and metabolism

Once absorbed in the tissue, the biochemical composition and physiological condition of the mussels affect the distribution and fate of the toxicants (Widdows & Donkin, 1992). For lipophilic compounds, there is a general correlation between lipid concentration in the organism or in a specific tissue and the degree of bioaccumulation of the compound. This would indicate that the toxicants associate to lipid stores in the tissue (Farrington, 1989; Livingstone & Pipe, 1992; Phillips, 1993). Factors affecting lipid levels, including seasonal storage cycles and metabolic activities, are therefore likely to influence bioaccumulation. In mussels, the digestive gland is the most lipid-rich organ (De Zwaan & Mathieu, 1992) and this is the major site for accumulation of both organic pollutants and OA (Yasumoto *et al.*, 1978; Livingstone & Pipe, 1992). Thus, a correlation between lipid content in the digestive gland and concentration of OA may exist which would partially explain the large variability in concentration of OA among individual mussels (Edebo, 1988) as well as seasonal differences in accumulation of OA. This model was investigated in **paper IV**.

Apart from associating to lipid-rich components in the tissue, toxicants may have more specific targets where the lysosomes are a common site for accumulation of many pollutants in mussels. Cells of the digestive gland of mussels are extremely rich in lysosomes (Moore, 1990) and storage within the lysosomes represents a protective mechanism against toxic effects of various compounds in mussels (Moore & Willows, 1998). However, accumulation of toxicants in high concentrations may cause structural and functional alterations of the lysosomes, thus harming the cell and the organism (Moore, 1990; Cajaraville *et al.*, 1995). The most common response to pollutants is a change in membrane stability due to enlargement of the lysosomes. This causes leakage of lysosomal components including the toxicant into the cytosol of the cell (Cajaraville *et al.*, 1995; Lowe *et al.*, 1995). In **paper II**, the *in vitro* effects of OA on the lysosomal compartment in mussel blood cells were studied.

Moore & Willows (1998) have proposed a model to illustrate how particulate-bound pollutants are taken up into the cells via vesicular transport to the lysosomes. This model may also apply for cellular uptake of OA *in vivo*, since OA is likely to associate to fragments of the digested algae. For toxicants accumulating within the lysosomes, factors which affect the dynamic processes of this system, for example digestive activities, could in theory influence the degree of bioaccumulation and depuration (**paper III**).

Whether a toxicant is metabolised or not influence bioaccumulation. The metabolites may have rates and routes of elimination different from the parent compound. For the individual, biotransformation should preferentially lead to the formation of a less toxic compound which can be excreted to the external environment. In mussels, biotransformation of organic pollutants by enzymes of the mixed-function oxygenase (MFO) and cytochrome P-450 systems occur, although the levels and activities of these systems are lower compared to vertebrates (Livingstone *et al.*, 1992; Moore & Willows, 1998). Biotransformation of OA to acyl-OA

(DTX-3) is regarded as taking place within the bivalve digestive gland since the DTX-3 compounds are generally not detected in the toxin-producing algae (see *1.2.4*). The formation of DTX-3 represent a detoxification of the parent compound since this molecule has low affinity for the protein phosphatases (Yanagi *et al.*, 1989; Yasumoto *et al.*, 1989; Takai *et al.*, 1992). DTX-3 is, however, a more hydrophobic compound compared to OA and should theoretically be more difficult to excrete. The enzyme systems responsible for acylation in mussels remain unidentified and the proportion of DTX-3 compared to OA is generally low in *Mytilus* sp. (Marr *et al.*, 1992; Fernández *et al.*, 1996b; Fernández *et al.*, 1998; Suzuki & Mitsuya, 2001; Vale & Sampayo, 2002b). This would indicate that biotransformation is not an important route for detoxification and elimination of OA in mussels (but see *3.2*, table 1).

1.5.3. Depuration

Depuration of hydrophobic contaminants is thought to be essentially a passive process in mussels, involving diffusion of the toxicant across membranes of different organs (Livingstone & Pipe, 1992). Elimination pathways include direct excretion across the gills and excretion from the digestive system by faecal deposition via the kidneys in the urine. In some cases, lipophilic compounds may also be eliminated through egg deposition during spawning. Since bioaccumulation is a function of uptake versus depuration rate, a change in one of these rates processes affects the level of toxicants in the tissue. A net loss can only occur if the uptake rate is lower than depuration rate (Spacie & Hamelink, 1985). Although depuration is mainly assumed to be a passive process, rates of elimination of pollutants are affected by factors such as season (probably via lipid levels) and temperature (Livingstone & Pipe, 1992).

When depuration rates and kinetics of toxicants are to be quantified, it is essential that the organism is not exposed to the toxicant or toxic algae, or that the uptake rate of toxicants is simultaneously quantified. Therefore, data on depuration from field observations are difficult to interpret since it is generally not possible to control the water column for toxicants. Experimental laboratory work is therefore normally used to study these issues. Compartment models can be useful tools to describe the processes of uptake and elimination of toxicants (Spacie & Hamelink, 1985; Silvert & Cembella, 1995). Depuration curves for most lipophilic contaminants are either exponential or biphasic which corresponds to a one-compartment and two-compartment model respectively. The one-compartment model assumes that the toxicant resides in the tissue in a common pool, equally available for depuration, and the rate of depuration is constant. In this case, data are accurately fitted to the exponential loss equation ($T_t=T_0*e^{-\lambda t}$ where T_0=concentration of toxicant at the start of depuration, λ=exponential decay coefficient, t=time) which can be used to calculate depuration half-lives ($t_{1/2}$) (**paper VI, V**). A two-compartment model assumes that toxicants are partitioned into two compartments with different rates of depuration within the animal. This model is characterized by a noticeable change in slope of the depuration curve. Depuration kinetics of lipophilic contaminants are markedly affected by the duration of pre-exposure to the contaminant where short-term exposure results in a rapid and complete elimination (one-compartment model) as opposed to long-term exposure where depuration is slower and often incomplete (two-compartment model; Livingstone & Pipe, 1992).

This may also apply for depuration of OA where observations on depuration kinetics in mussels suggest that both exponential and biphasic depuration models are possible (Marcaillou-Le Baut *et al.*, 1993; Croci *et al.*,

1994; Blanco *et al.*, 1995; Fernández *et al.*, 1998; Blanco *et al.*, 1999 and **paper III**, **IV** and **V**). Also, depuration half-lives reported for OA in mussels vary considerably. Although the digestive gland is the major storage organ of OA, there is a lack of more detailed information about cellular or intracellular localisation of OA which would help in understanding the elimination processes of this toxin (see *3.1*). There is also a general lack of knowledge about the influence of environmental factors on the depuration dynamics of this algal toxin although field observations have correlated the rate of depuration to the availability of food in the water (Haamer, 1990a; Sampayo *et al.*, 1990; Marcaillou-Le Baut *et al.*, 1993; Poletti *et al.*, 1996; Blanco *et al.*, 1999). Blanco *et al.* (1999) proposed a physiological process model to explain how feeding and digestion affects the mechanism and rate of depuration of OA. This theory predicts that increased digestive activities concurrently accelerate the rate of metabolic faecal loss, including the elimination of OA through faecal deposition (Blanco *et al.*, 1999). The route for elimination of OA in mussels is thought to be via faecal deposition (Blanco *et al.*, 1999) however, there is no direct evidence for this. If this model is valid, then the amount of ingested food should be positively correlated to the rate of OA depuration. This hypothesis was experimentally tested in **paper III**. The influence of environmental factors (food and temperature) on rate of depuration of OA was also studied in **paper IV** and a large-scale field depuration experiment was conducted in **paper V**.

1.6. Objective of this thesis

The main objective of this thesis has been to improve the understanding of how mussels survive exposure to OA and how this algal toxin is eliminated from the mussels, including the influence of external factors on this process. More specifically, I have tried to answer the following questions:

- Do mussels have mechanisms which protect the cells against the harmful effects of okadaic acid (**paper I** and **II**)?
- Which physiological mechanisms regulate depuration and elimination of okadaic acid (**paper III** and **IV**)?
- Which environmental factors affect the rate of depuration of okadaic acid in mussels?
- Is it possible to accelerate depuration by manipulating environmental conditions (**paper III** and **IV**)?
- Is large-scale field depuration a management option for the industry (**paper V**)?
- Does species-specific accumulation of DST exist among bivalve species in Swedish waters (**paper VI**)?

2. Detection methods

This section briefly discusses the analytical detection methods for DST which have been used in this thesis. A short description of the mouse bioassay is included since this is the official detection method for monitoring purposes within the European Union.

2.1. Mouse bioassay

The mouse bioassay is probably the most widely used analytical test to detect various types of shellfish toxins (Andersen, 1996). This assay dominates the analysis of PSP toxins for consumers protection (Jellet, 1993; Fernández *et al.*, 1996a). The mouse assay was also the first test developed to monitor the DSP toxins in shellfish extracts (Yasumoto *et al.*, 1978). The main principle of the mouse bioassay is to inject a sample of shellfish extract intraperitoneally into a mouse and measure the time to death, which is ideally directly related to the amount of toxin in the sample calculated from a standard curve. One mouse unit (MU) in the DSP mouse assay was originally defined by Yasumoto *et al.* (1978) as the minimum dose of toxin to kill a mouse within 48 hours. Mouse units have lately been redefined as the amount of toxin required to kill two of three mice (20 g) in 24 hours, where one MU corresponds to 4 µg OA, 3.2 µg DTX-1 and 5 µg DTX-3 (Yasumoto *et al.*, 1995). The current upper limit for marketing of shellfish within the European community is 160 µg OA*kg^{-1} mussel meat. This corresponds to the detection limit in the mouse bioassay, thus if the mice react to the sample, then it is considered above the regulation limit.

A critical step for the outcome of the mouse assay is the extraction method chosen and various modifications of the Yasumoto extraction protocol are commonly used (James *et al.*, 2000). The extraction should preferably result in a sample which contains all DST derivatives but not interfering compounds such as other toxins or lipids. The main advantage of the DSP mouse bioassay is that it measures the total toxicity in the sample, including potentially unknown toxins, and therefore serves as a good control method for monitoring purposes. However, this method has several disadvantages. For example, it is time-consuming and provides no information as to which individual members of each toxin families are present. In addition, it can generate false positives, as the mouse reacts to endogenous compounds in the mussels such as free fatty acids (Suzuki *et al.*, 1996). The method is also less sensitive than other chemical detection methods or *in vitro* bioassays. Apart from the methodological problems with the mouse bioassay, the ethical aspects of using live animals in toxin testing are of great concern and alternative methods are used in several countries (Fernández *et al.*, 1996a).

2.2. High performance liquid chromatography (HPLC)

HPLC is widely employed as a sensitive and selective method to detect DSP toxins in shellfish samples (Lee *et al.*, 1987; Edebo, 1988; Gago *et al.*, 1996; James *et al.*, 2000). The first HPLC method was developed by Lee *et al.* (1987) and is based on the coupling of a fluorescent reagent (9-anthryldiazomethane; ADAM) to the carboxylic group of the DST, enabling fluorometric detection. The initial extraction procedure is done in 80% methanol, followed

by a cleanup step using petroleum ether. The toxins are then extracted into a chloroform phase. The chloroform is evaporated and the derivatization with ADAM is conducted. The sample is further cleaned using a silica solid phase extraction (SPE) cartridge. Isocratic HPLC separation on a reverse-phase column (octadecylsilane; ODS) coupled to a fluorescence detector is the final step in this method. The toxin concentration in the shellfish extract is calculated from a standard sample which is run in parallel to the extracts. OA, DTX-1 and DTX-2 are directly detected by this method but the acylated forms of DST (DTX-3) can only be analysed after removal of the acyl group, converting the DTX-3 to its respective parent compound. Initially, a different sample clean-up process to retain the low polarity DTX-3 in digestive gland extracts is necessary. A hydrolysis step then follows in order to remove the acylated portion of DTX-3. This produces the respective DST parent compounds. The amounts of DST parent compound in the extracts before and after hydrolysis are analysed, providing a measure of the proportion of DTX-3 present in the tissue. Detailed descriptions of the analytical steps to detect DTX-3 have been published by Lee *et al.*, (1989); Marr *et al.*, (1992); Fernández *et al.*, (1996b); Suzuki *et al.*, (1998); Vale & Sampayo, (2002b).

Various modifications of the HPLC method according to Lee *et al.* (1987) have been published in order to improve both the extraction procedures and post-derivatization sample clean-up. In addition, different fluorimetric derivatizing reagents have been employed (James *et al.*, 2000). In the Swedish monitoring program, HPLC with slight modifications according to Edebo *et al.* (1988) is routinely used for detection of OA and DTX-1. 1-pyrenyldiazomethane (PDAM) is used instead of ADAM in Sweden since it is proven to be a more stable compound than ADAM. The detection limit is 50 ng OA*g^{-1} mussel meat. HPLC was used in **paper III** and **V** to analyse OA and DTX-1 in mussel extracts.

Liquid chromatography coupled with mass spectrometry (LC-MS) is the most powerful chemical method today to identify and characterize toxins, especially previously unknown compounds (Marr *et al.*, 1992; Quilliam, 1995; Draisci *et al.*, 1995) but the equipment is highly sophisticated and expensive and not yet available in most laboratories involved in biotoxin analysis.

2.3. Protein phosphatase inhibition assay (PPIA)

Although HPLC is sensitive enough for monitoring purposes, it is a relatively expensive and time-consuming method and alternative detection methods which are cheaper, more rapid and sensitive are desired. A number of protein phosphatase inhibition assays (PPIA) using purified protein phosphatase 2A have been developed as satisfactory alternatives to conventional methods (Honkanen *et al.*, 1996; Tubaro *et al.*, 1996a; Vieytes *et al.*, 1997; Shimizu *et al.*, 1998). These assays measure the catalytic dephosphorylation activity of PP2A on various substrates having a phosphate group attached to the molecule. The release of the phosphate group by the action of PP2A generates a product which can be detected in several ways depending on the substrates. Assays employing radioactivity, colorimetry, bioluminescence or flurorometry as detection modes are described but the methods based on coloremitry and fluorometry seem to be preferentially used in practice. Because PP2A activity is inhibited by low amounts of DST (OA, DTX-1 or DTX-2 but not DTX-3), the addition of any of these toxins to the PPIA inhibits the release of the phosphate group in a dose-dependent manner. The amount of toxins in shellfish extracts can be quantified using a standard curve. Since this method does not distinguish between individual

toxins but measures the total inhibitory activity in a sample, the amount of toxin in a sample is expressed as OA equivalents when OA is used as a standard material. Quantification of DTX-3 in PPIA can be done using the same steps as described for HPLC (see *2.2.*), i.e. alkaline hydrolysis to release the acyl group from DTX-3 with subsequent detection of the parent compound. Mountfort *et al.* (2001) have adapted the PPIA for determination of total DSP content in shellfish samples.

Comparative evaluations of the colorimetric and fluorometric PPIA have shown that there is generally a good agreement between this method and other quantitative detection methods for DST (Della Loggia *et al.*, 1998; Fontal *et al.*, 1998; Mountfort *et al.*, 1999; Ramstad *et al.*, 2001). Detection limits in shellfish extracts range from 1 to 10 ng OA*g^{-1} digestive gland which makes this method about 100-200 times more sensitive than HPLC (Tubaro *et al.*, 1996a; Vieytes *et al.*, 1997; Mountfort *et al.*, 1999; Ramstad *et al.*, 2001). Apart from increased sensitivity, the main advantage of the PPIA assay is that a large number of samples can be processed and measured simultaneously in 96-well microplate readers, making it suitable for screening of larger samples. It is a very useful method in experimental work comparing toxicity of samples among experimental treatments. The use of microplates also means that very small sample volumes can be used and the cost per sample of the enzyme and other chemicals are kept to a minimum. PP2A is, however, a relatively expensive product and experiences from our lab indicate that the activity of the enzyme may vary between batches. A modification of the fluorometric PPIA assay developed by Vieytes *et al.* (1997) was applied to quantify DST in mussel and algal extracts in **paper VI**, **V** and **VI** (Godhe *et al.*, 2002). Compared to Vieytes *et al.* (1997), we have found that the initial 80% methanol extract needs to be further cleaned to remove non-specific inhibitory compounds from the mussel extract. We use the sample clean-up method for HPLC by Lee *et al.* (1987). Provided that this additional clean-up step is conducted, this method has been very accurate and highly suitable for application in the experimental work presented in this thesis.

3. Results and discussion

This section summarizes the most important findings and conclusions from the papers included in this thesis.

3.1. Intracellular responses and protective mechanisms

In **paper I** and **II**, studies on the intracellular effects and potential protective mechanisms against toxic effects of OA in mussels were performed. In **paper I**, the inhibitory effects of OA on protein phosphatase 1 (PP 1) and PP 2A activities were measured in non-toxic and toxic mussel tissue extracts (see*1.3.1*). This was done to test the hypothesis that mussels expressed structurally modified PP which were insensitive to inhibition by OA. Rainbow trout tissue was used as a comparison and represented an animal which does not naturally experience OA. Mussel PP were found to be just as sensitive to *in vitro* inhibition by OA as rainbow trout PP and other organisms studied so far. Thus, modification of the molecular structure of PP did not explain the apparent resistance against OA in mussels. There were also indications that PP activities in extracts from toxic mussels were inhibited to some degree which suggested that OA may cause secondary changes in the phosphorylation state of enzymes in mussels. The enzyme glycogen synthase (GS) was then used as a model system to study the secondary effects of OA on a cytosolic enzyme, whose activity is regulated by reversible phosphorylation (see *1.3.2*). GS activity has been identified and characterized previously in *M. edulis* (Cook & Gabbot, 1978; Gabbot, 1986; Whittle & Gabbot, 1986). Glycogen is also the major energy storage product in blue mussels (De Zwaan & Mathieu, 1992), thus disturbances of the glycogen metabolism by OA may affect the overall fitness of the individual mussels. An *in vitro* tissue slice technique, modified after Whittle & Gabbot (1986), was used where thin slices of digestive gland or rainbow trout liver were incubated in 1-10 µM OA and the activity of GS was measured after 4 to 6 hours of incubation. GS activity was not significantly inhibited by 10 µM OA in mussel tissue, however, a substantial reduction in GS activity in the presence of 10 µM OA was detected in rainbow trout liver. An *in vivo* experiment was performed where mussels were fed the OA-producer *Prorocentrum lima* or the non-toxic species *P. micans* for 8 days. OA content and GS activity was measured after 2 and 8 days. Mussels feeding on *P. lima* accumulated concentrations of OA corresponding to levels that occur naturally in populations of mussels. These amounts of OA was also theoretically estimated to fully inhibit the activity of PP in the tissue. Even so, there were no indications of inhibition of GS due to the presence of OA. The main conclusions from **paper I** were that OA is prevented from binding to PP in the cells of the digestive gland. It was suggested that uptake of OA into the lysosomal compartment could represent a mechanism that protects the rest of the cell from the cytotoxic effects of this toxin. As discussed in the introduction, cells of the digestive gland in mussels are particularly rich in lysosomes, involved in intracellular digestion of food particles (see *1.4.2*) and sequestering of xenobiotics and heavy metals (see *1.5.2*). Also, mussels have been shown to have limited ability to metabolise pollutants, thus a highly developed lysosomal system is a way for these organisms to resist harmful effects of such compounds (Moore, 1990).

To follow up on the role of the lysosomal system during OA exposure, we emphasized the importance of identifying the cellular and intracellular storage site of OA in **paper I**. Several attempts to use immunohistochemical techniques to localize OA in mussel tissue have been carried out during this Ph. D. period. Both a commercial mouse monoclonal antibody produced by Rougier BioTech, Montreal (Svensson 1995, Honours thesis) as well as a polyclonal antibody against OA raised in rabbit (generously provided by J. Cordová) has been tested on digestive gland slices from toxic mussels in a variety of different fixatives and incubation protocols, however, this technique was never successful. An alternative approach to identify intracellular storage sites is to use centrifugation techniques to isolate subcellular organelles in toxic digestive gland tissue and then measure concentrations of OA in each fraction. Some methodological adaptations of the subcellular isolation techniques for mussel tissue are, however, needed and this was beyond the time schedule of this Ph. D. The tissue slice technique used in **paper I** was concluded to be a simple and very useful system to carry out controlled *in vitro* experiments to test hypothesis about effects and mechanisms in the digestive gland during OA exposure. This method is recommended for future studies.

In **paper II**, mussel blood cells were used as a model system to investigate the cytotoxicity and cellular responses of OA. This cell system has several advantages, for example, primary cultures of blood cells are easily isolated by extracting hemolymph from the posterior adductor muscle using a syringe. The hemolymph of mussels do not contain oxygen-carrying ("red") cell types. Only "white" blood cells involved in digestion and internal defence are present. This means that no separation of red cells from the hemolymph is necessary prior to use. The blood cells quickly attach to surfaces of e.g. Petri dishes where incubations in various compounds can be made. Subsequent separation from the overlying solutions and washing steps are therefore easy to carry out which is another advantage with these cells. A primary culture of digestive gland cells has also been used to test the cytotoxicity of OA. Cells were isolated according to Birmelin *et al.* (1999). Dissection and dissociation of the digestive gland to obtain a cell suspension is time-consuming and these cell types do not attach to surfaces which means that a centrifugation step has to be included each time medium is separated from the cells. These cells are also less robust compared to blood cells and the mortality of digestive cells is high during the first days of culturing (70% mortality after three days; Birmelin *et al.*, 1999). Thus, cytotoxicity studies, which should be carried out within three days, become difficult with digestive cell cultures. On the other hand, the relevance of using digestive cells to study effects of OA is higher compared to circulating blood cells since the digestive gland is the major site of OA accumulation *in vivo*.

The toxicity of OA in blood cells was investigated by incubating the cells in increasing concentrations of OA (1 nM-1 µM) for 24-72 hours. The proportion of living cells to total cell number (viability) was estimated by dye exclusion, using the red dye Eosin-Y, and light microscopy. Viable cells do not accumulate this compound but non-viable cells are stained and easily recognised as red cells under the microscope, hence, this method is a direct measure of viability. Mussel blood cells were found to be extremely resistant against the cytotoxic effects of OA compared to results published for other cell types. For example, we observed no reduction in viability in 1-100 nM OA compared to control cells, only 1 µM OA had an effect on viability (54% after 72 hours). This meant that the LC_{50} value, which is the OA concentration that reduces the number of viable cells to 50%, was >1 µM in these cells. For other cell types, which include

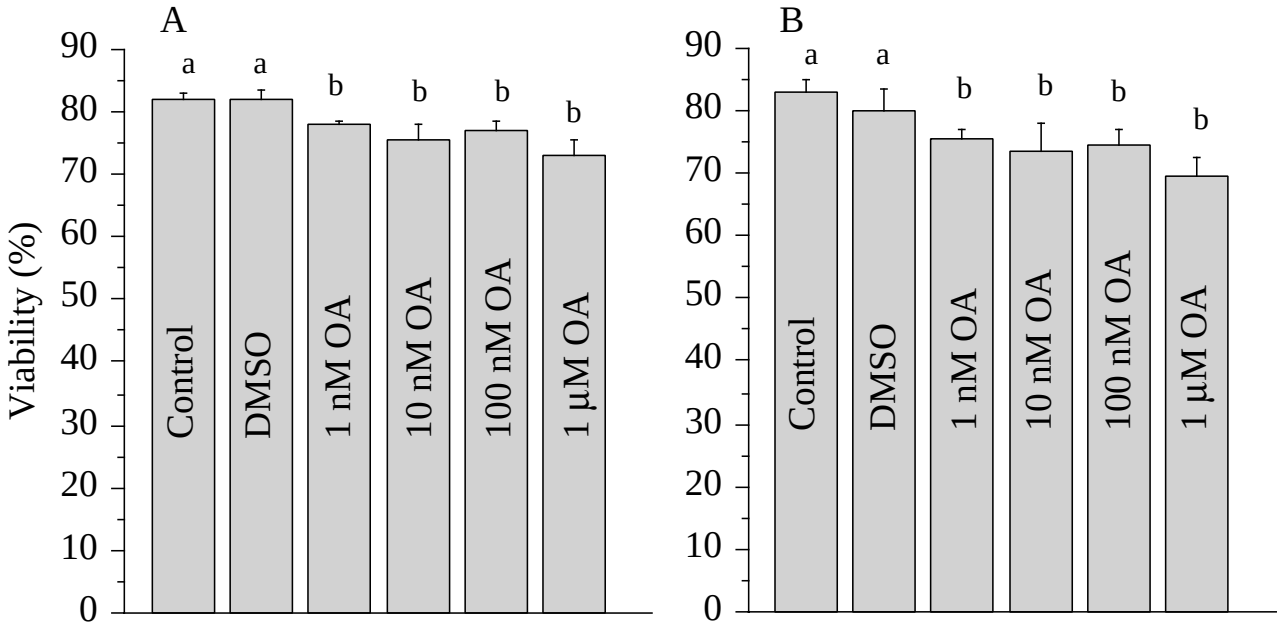


Fig. 4. Viability of primary cultures of digestive gland cells after exposure to various concentrations of OA for 12 hours. A: Small (agranular) cells B: Digestive (granulated) cells.

both mammalian and fish cell lines, LC_{50} values for OA range from 1.5 nM to 31 nM (*1.3.3*). Thus, mussel blood cells can resist 30-1000 times greater concentrations of this compound. Preliminary results from the study on the toxic effects of OA in mussel digestive gland cell cultures suggest that these cells are less resistant to OA compared to blood cells (fig. 4, unpublished results). Already after 12 hours of incubation, viability was significantly reduced in all concentrations of OA compared to control cells.

The resistance against OA was proposed to be due to multixenobiotic resistance (MXR), or p-glycoprotein (p-gp) activity, in the blood cell membranes (see *1.3.4*). To test the effects of OA, a functioning assay to characterise general cell membrane p-gp activity in these cells was needed. Two different substrates of p-gp activity was tested; the *Vinca* alkaloid Vincristine (VCR), radiolabelled with ^{3}H, and the fluorescent substance Rhodamine B (Rh B). Also, two different inhibitors of p-gp activity were used in the assays; the calcium-channel blocker Verapamil (VP) and the protein kinase C inhibitor Staurosporine (ST). These are well-documented substrates and inhibitors of p-gp activity in many cell systems. Although repeated experiments using various concentrations of substrates and inhibitors and different incubation protocols were performed, it was not possible to confirm that mussel blood cells expressed so-called "classical" p-gp activity in the cell membranes (fig. 5A). A classical p-gp activity response predicts that the efflux of p-gp substrates is inhibited in the presence of VP which is considered evidence of cell membrane p-gp activity (Bard, 2000). Instead, the opposite was observed, that is, the intracellular accumulation of both VCR and Rh B was reduced by VP. These results were in contrast to (Minier & Moore, 1996) who found that *M. edulis* blood cells expressed functional p-gp in the cell membranes. Since these results were puzzling, additional experiments using mussel gill tissue to serve as a positive control for the assay system were performed. P-gp expression and activity has been identified and is well characterised in *Mytilus* sp. gill tissue (Minier *et al.*, 1993; Cornwall *et al.*, 1995; Kurelec, 1995). It was found that the intracellular accumulation of Rh B increased in the presence of VP in a dose-dependent manner, confirming cell

membrane p-gp activity in this tissue as well as the accuracy of the assay. The addition of OA to gill tissue similarly increased Rh B accumulation which suggested that OA is either a competitive substrate or inhibitor of p-gp activity.

A model which would explain the observations made in the blood cells was proposed (fig. 5B). It was suggested that the main site for p-gp-related activity in these cells were within subcellular organelle membranes such as the lysosomes. P-gp substrates enter the cytosol via passive diffusion through the cell membrane. Then, p-gp mediated transport from the cytosol across the lysosomal membranes into this compartment occurs. Thus, substrates of p-gp are actively concentrated within the lysosomes against a concentration gradient. The addition of p-gp inhibitors interferes with the lysosomal accumulation which explains why intracellular concentrations of VCR and Rh B were reduced by VP and ST in our experiments. This pattern was also directly confirmed when cells were observed under a fluorescence microscope. Accumulation of Rh B within lysosomes was visually observed under microscope and the lysosomal fluorescence intensity was markedly reduced in cells treated with VP. Because mussel blood cells are involved in internal defence and are highly rich in lysosomes, this model should go some way in explaining how the organism is protected from deleterious effects of various toxic compounds. The possibility of intracellular MXR transport as a mode of lysosomal accumulation of environmental pollutants has previously been discussed by Moore & Willows (1998). Likewise, the lysosomes are the main site of p-gp expression in cells of crab (*Carcinus maenas*) hepatopancreas (Köhler *et al.*, 1998).

Whether OA is actively transported by p-gp activity into the blood cell lysosomes is unclear from this study. However, when the volume of the lysosomal compartment was measured in cells pre-exposed to increasing

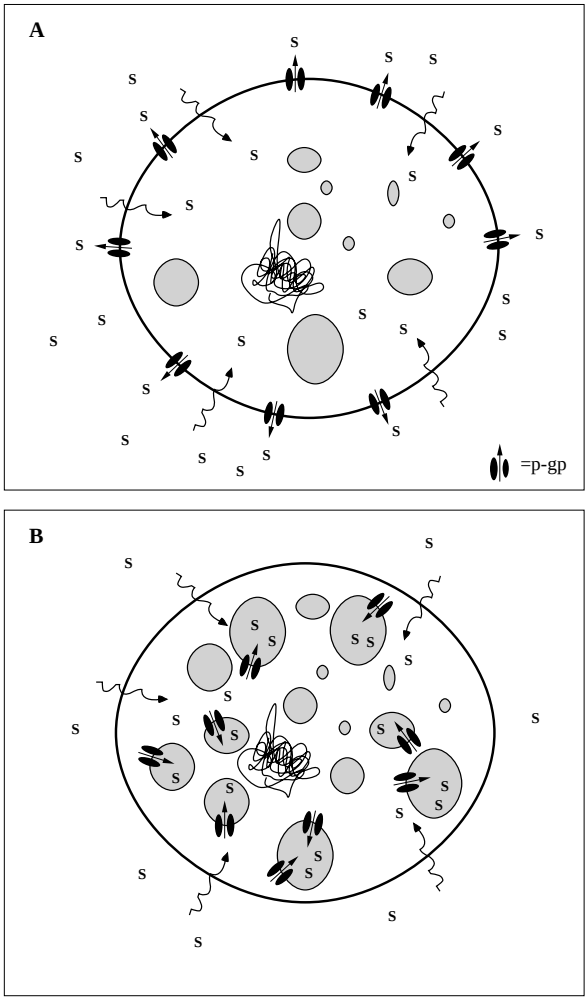


Fig. 5. Simplified models of the function of p-glycoproteins (p-gp). A. “Classic” multidrug resistance response. The main location and activity of p-gp is in the plasma membrane. Substrates (S) enter the cell through passive diffusion (crooked arrows). In the cytosol, substrates are actively transported by p-gp to the external environment against a concentration gradient. B. Hypothesized function of p-gp in mussel blood cells (**paper II**). The main location and activity of p-gp are within lysosomal membranes. P-gp substrates diffuse through the plasma membrane into the cytosol where transport into lysosomal organelles by p-gp-like activity occurs.

concentrations of OA, it was observed that the lysosomal volume increased accordingly in 10-100 nM OA but to a lesser degree in 1 µM OA compared to control cells. This indicated that OA accumulated within this compartment which caused swelling of the lysosomal organelles. It is likely that enlargement of the organelles reduced lysosomal membrane integrity in the presence of 1 µM OA, causing

leakage of lysosomal components into the cytosol including OA. It is well established for organic micropollutants and toxic metals accumulating within the lysosomal system that these may cause membrane damages which is a significant factor in cell injury (Moore & Willows, 1998) and (*1.5.2*). Thus, structural changes in the lysosomal membranes with subsequent failure to retain OA within the lysosomes could explain the cytotoxic effects of 1 µM OA, but not 1-100 nM, as observed in this paper. To conclude, the overall results from **paper II** indicate that the lysosomal system may be involved in protecting mussel blood cells from the harmful effects of toxicants such as OA. The importance of this system during *in vivo* exposure to OA in mussels remains to be investigated in future studies.

3.2. Depuration mechanisms and influence of environmental factors

The general aims of **paper III** and **IV** were two-fold; (1). to test hypotheses about systems involved in the elimination of this toxin from a physiological viewpoint and (2). to test the effectiveness in a management perspective of manipulating environmental conditions in order to accelerate the depuration rate of this toxin. In **paper III**, the importance of feeding and digestion for elimination of OA was investigated. It has been postulated in literature that the main environmental factor regulating the rate of depuration of DST is the quantities of nontoxic food available for mussels to feed upon in the water mass (see *1.5.3*). This originates from observations on depuration in natural populations of *Mytilus* sp. where a positive correlation between the concentration of phytoplankton and elimination rate of DST has been found. This relationship has not previously been tested in a manipulative experiment. A physiological model which would explain how food affects the mechanism of depuration in mussels was proposed by Blanco *et al.* (1999). Ingestion rates increase when food becomes more abundant resulting in higher digestive activities and subsequently greater metabolic faecal losses. OA assimilated in the digestive gland tissue is released in this process and eliminated in faeces. This model should be relevant if the toxin is associated to components of intracellular digestion, then it seems likely that elimination of OA would be accelerated during periods of high digestive activities. In **paper II,** it was speculated that OA might be stored within lysosomes of the digestive cells. Considering the role of this subcellular compartment in digestion of food, increased uptake and excretion of end-products of intracellular digestion could also facilitate OA depuration.

This model was tested in a laboratory experiment where toxic mussels from a long-line farm were fed different rations of an algal mixture during a period of 32 days. It was predicted that the concentration of OA would decrease at a faster rate in mussels feeding on high amounts of algae compared to lower amounts and those subjected to starvation. The production of faeces was used as an indirect measure of ingestion and changes in body mass were monitored during the experiment. A static system was used where individual mussels were kept in buckets with filtered and aerated seawater to which known amounts of algae were manually added. The static system was chosen instead of a flow-through for several reasons, the most important being the possibility to control the algal rations ingested by each individual since all cells added to the buckets were filtered by the mussels. This becomes much more difficult in a flow-through system where the actual clearance rate needs to be measured for each individual in order to calculate ingestion. Also, faecal pellets were easy to collect in the buckets and the qualitative difference among treatments in faecal production indirectly

confirmed that mussels were ingesting algae according to the intentions.

A significant effect of food treatment was detected after four days of depuration, where OA concentration was higher in mussels receiving no food compared to feeding mussels. OA concentration actually increased in all treatments during the first days of depuration and this increase was most pronounced in the no food treatment at day four. It was tested if this apparent increase in OA concentration could be due to a simultaneous reduction in the digestive gland mass. Total content of OA (concentration of OA*digestive gland mass) was used as a variable. The second statistical analysis did not detect any differences among treatments, thus it was concluded that it is important to monitor changes in tissue mass to be able to interpret results from depuration experiments correctly. A general temporal reduction in OA content was observed during the experiment regardless of whether the mussels were feeding or not with an average half-life of 16 days. In contrast to the predictions, a strong trend towards lower levels of OA in mussels subjected to food limitation compared to feeding mussels was observed at the end of the experiment. Mussels not receiving food appeared to lose a large proportion of OA between day 16 and day 32 whereas OA content in feeding mussels remained unchanged between these sampling times. This loss of OA correlated to a substantial loss of body mass in the no food treatment which indicated that mussels were affected by starvation. To explain the findings that OA appeared to be eliminated at a faster rate in starving mussels, it was speculated that a proportion of OA might have affinity for lipid-rich cellular or intracellular components such as lipid droplets or membrane structures in the digestive gland tissue (see *1.5.2*). The release of OA from this compartment would in that case be facilitated by increased usage of such components and an increased demand for lipids as an energy source is likely to occur during food limitation.

From the results in **paper III**, it was concluded that elimination of OA is not dependent on digestive activities and occurs regardless of whether the mussels are feeding or not. This is somewhat contradictory to the predicted effects for those substances residing within the lysosomal system (Moore, 1990; Moore & Willows, 1998). A plausible explanation could be that these compounds attach to lysosomal components such as membrane structures or lysosomal pigments which are recirculated and not excreted from the cells during digestion.

As discussed earlier in this section and in section *1.5.1* and *1.5.3*, field observations indicate that depuration rates of DST increases in conjunction with the increased availability of nontoxic phytoplankton. In **paper III**, a model was proposed which could explain this pattern in the natural situation. This model suggests that the quantity of food affects the mechanism of ingestion and/or absorption of toxins rather than directly affecting the depuration mechanisms. Bioaccumulation of compounds in an organism is a function of uptake and depuration rate (see *1.5.1*). A change in one of these variables would either increase or reduce bioaccumulation. For OA, the principal route of uptake in mussels is by feeding on toxin-producing dinoflagellates. The resulting assimilation and uptake of the toxins into the tissue takes place within the digestive system. If the ingestion of toxic algae per time unit is reduced, then this could result in a net loss of toxin from the organism even though the depuration rate remains constant. Alternatives which could result in a reduced ingestion of toxic algae include (1): The absolute concentration of toxic dinoflagellates decreases or the algae disappears from the water column, filtration rate is maintained in the mussels (2): The relative abundance of accompanying nontoxic species increases which alters the filtration

and ingestion rates of the mussels. Similar result could also be obtained if the absorption of the toxin within the digestive system is reduced. Absorption efficiency has been found to decrease mainly as a result of faster gut passage times during periods of high ingestion rates in mussels. Thus it seems likely that absorption of OA is reduced when the rates of feeding and ingestion are high. Numerous studies have provided convincing evidence that feeding behaviour and nutrient acquisition of *Mytilus* sp. are highly regulated by the phytoplankton concentration in the water mass (Hawkins & Bayne, 1992). In this context, feeding affects elimination of OA but not by regulating the actual depuration mechanisms.

Some general conclusions from **paper III** regarding depuration as a management option were drawn. Since depuration of OA was not accelerated in feeding mussels, potential depuration methods without addition of extra food, reducing the cost for depuration, could be considered. Long periods of starvation are, however, not recommended due to the negative effects on the condition of the mussels.

In **paper IV**, the model proposed in **paper III** regarding the importance of lipid turnover for elimination of OA was investigated. Initially, a field study was performed to explore whether there is evidence of a relationship between lipid content and OA in the natural environment. Mussels from a long-line farm were sampled once a month from January to June, 2000, and OA concentration, together with total lipid content in the digestive gland, were analysed in individual mussels. From January to March, a substantial decrease in OA concentration occurred and the same trend was observed for lipid content during this period. The significance of this correlation was analysed and a positive relationship between these variables was detected. This supported the proposed model that increased usage of lipid stores could accelerate the elimination rate of OA. It should be noted that an increase in lipid content was observed during the last sampling occasions (April to June) whereas OA concentration continued to decrease. This suggested that the release of this toxin is not exclusively governed by rate processes involved in uptake and metabolism of lipids. Data on OA in mussels together with plankton concentration in the farm site area (national monitoring program) were also compiled in order to study the temporal relationship between OA depuration and blooms of phytoplankton. The largest reduction in OA concentration in mussels occurred between January and February, before the peak of the diatom spring bloom, which occurred between February and March during this season. Thus, feeding did not seem to be a prerequisite for depuration of OA to occur, a conclusion which is in line with the results from **paper III**.

A laboratory experiment was performed to test the relationship between turnover of lipids and elimination of OA. In order to do this, it was needed to obtain variability in lipid content among individuals or groups of toxic mussels during the experiment. Therefore, factors which could influence the energy requirements and need to metabolise lipids were considered. In poikilothermic organisms such as mussels, the energy requirements for maintenance metabolism is strongly regulated by the surrounding water temperature (Wieser, 1973; Hawkins & Bayne, 1992). In *Mytilus* sp., metabolic rate processes and usage of body energy reserves are also highly regulated by the availability of food (Hawkins & Bayne, 1992). By exposing mussels to an increased sea water temperature and/or food limitation, it was predicted that the energy demand and lipid metabolism should increase compared to feeding mussels maintained in ambient temperature. This would then result in a faster depuration rate of OA, i.e. a positive correlation between lipid content and OA concentration was expected. Toxic mussels from a farm site were

depurated during 24 days in a flow-through system in either 18°C (ambient) or 24°C with or without the addition of algal food particles. Sampling was performed after 8, 16 and 24 days of depuration. The results showed that lipid content was significantly reduced in mussels exposed to 24° C compared to ambient water temperature already after 8 days in the experiment. Food limitation, on the other hand, did not reduce the amounts of lipids in the digestive glands compared to feeding mussels. It was speculated that food limitation could have caused a decline in metabolic rates (and hence energy requirements) which have been documented previously for *M. edulis* as a response to starvation (Bayne *et al.*, 1973; Widdows, 1973). Although the attempts to influence lipid levels by increasing the water temperature were successful, this did not increase the elimination rate of OA. No correlation between these variables was detected which was the opposite to the results from the field study. Since it was not possible to experimentally verify the significance of this relationship, the model that lipid metabolism affects the mechanism of elimination of OA was rejected.

Neither temperature nor food conditions had any effect on depuration rate of OA in this experiment, concentration of OA was similarly reduced in time regardless of treatments. An exponential decrease of OA was observed and depuration half-life ($t_{1/2}$) was calculated to 8 days. This is considerably faster than the rate observed in **paper III** ($t_{1/2}$=16 days). Likewise, depuration rates in *Mytilus* sp. published by Lindahl & Hageltorn (1986) and Marcaillou-Le Baut *et al.* (1993) indicate that the elimination rate of DST can be slow ($t_{1/2}$=1 to 1.5 months). Faster rates similar to those found in this experiment have been documented by Blanco *et al.* (1999; $t_{1/2}$=7-8 days), Fernández *et al.* (1998) ($t_{1/2}$=11-12 days) and Marcaillou-Le Baut *et al.* (1993) ($t_{1/2}$=12 days). Croci *et al.* (1994) reported very high rates for mussels depurated in ozonised water ($t_{1/2}$=3 days). Thus, rates of depuration differ greatly for both natural populations of mussels and in the laboratory environment.

A previous attempt to increase the depuration rate of DST by raising the prevailing water temperature was unsuccessful (Lindahl & Hageltorn, 1986). Also, the experimental results regarding the influence of food on depuration rate of OA presented in both **paper III** and **IV** indicate that depuration is not accelerated in feeding mussels compared to non-feeding mussels. In addition to the depuration experiments in **paper III** and **IV**, a separate laboratory experiment has also been performed where the effects of increased water temperature (from 12°C to 18°C) and reduced salinity (from 3.0 % to 1.8%) on rate of depuration of OA was tested (fig. 6, unpublished results). The OA concentration decreased independently of the surrounding environmental conditions and the average rate was highly similar to that observed in **paper III**.

To summarize, the results emphasize difficulties incurred in attempts to influence the mechanisms of depuration of DST in mussels through manipulation of external factors. This was also concluded by Morono *et al.* (1998). This indicate that processes

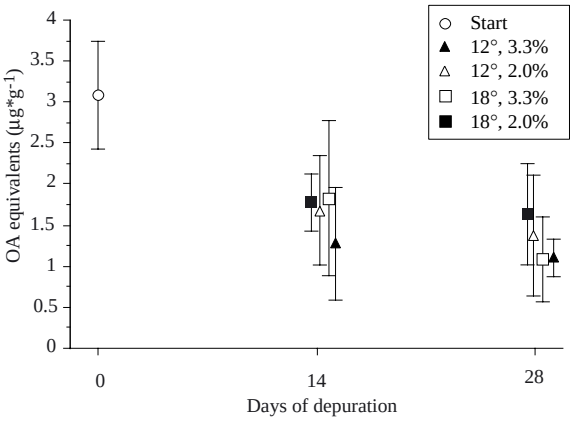


Fig. 6. Depuration of mussels in the laboratory, November 2000. Mussels were exposed to different combinations of temperature and salinity under conditions of food limitation. OA concentration was measured after 14 and 28 days of depuration.

insensitive to short-term changes in the environment are involved in regulating the elimination rates of these toxins. It seems possible that depuration is mainly a passive process (i.e. diffusive mechanisms) similar to what is known for other lipophilic toxicants (see *1.5.3*). Also, it was proposed that seasonal differences in the physiological status of the mussel, which may correlate to factors such as temperature and food, could explain some of the variability in depuration rates of toxins in mussels. For mussel populations in temperate latitudes, the physiological status is related to the annual reproductive cycle which is in turn associated with seasonal changes in both biochemical composition and physiological rate processes (Hawkins & Bayne, 1992). For example, a seasonality in the physiological response to external factors such as food have been documented for *M. edulis*. Hawkins & Bayne (1984) showed that, despite similar availability of the same algal species, rates of ingestion were less than half in a winter experiment compared to those measured during summer under otherwise identical conditions. There is also convincing evidence of an endogenous regulation of the seasonal balance between acquisition and utilization of nutrients with a considerably lower metabolic efficiency during winter compared to autumn (Hawkins & Bayne, 1984; Hawkins & Bayne, 1985). It seems possible that the rate processes involved in uptake and depuration of algal toxins show similar seasonal fluctuations. Indeed, slow depuration rates were observed in Swedish blue mussels during the winter season (**paper III,** Lindahl & Hageltorn, 1986) whereas faster rates were found during late summer (**paper IV** and **V**). Thus, season should be considered a relevant factor to include when uptake and elimination of toxins are studied.

Further research into the physiological and biochemical systems regulating bioaccumulation and elimination of the DST are needed and it still remains to identify the molecular binding site and storage location of DST in the mussel digestive gland tissue. Córdova *et al.* (2001) recently published results which suggested that endogenous PP are the binding target for OA in naturally contaminated mussels (*M. chilensis*) and clams (*Venus antiqua*). If so, the turnover rate of PP should affect depuration rate of OA. There were also some indications that the clam were able to compensate for the resulting loss in PP activity by increasing the expression and activity of a different isoform of PP, insensitive to inhibition by OA. This is an interesting observation which may identify a protective mechanism against cytotoxic effects of OA in shellfish frequently exposed to this algal toxin. This theory could explain the results in **paper I** where the activity of glycogen synthase was unaffected by OA even though there were indications of inhibited activity of PP1 and PP2A in naturally intoxicated mussels.

The scope of this thesis has not been to study the occurrence and magnitude of biotransformation of DST. There is now good evidence that conversion of OA, DTX-1 or DTX-2 to their respective acylated forms (DTX-3) take place in the digestive gland tissue of bivalves (see *1.2.4* and *1.5.2*) Large proportions of DTX-3 are commonly found in some bivalve species (Suzuki *et al.*, 1998; Suzuki & Mitsuya, 2001; Vale & Sampayo, 2002a; Vale & Sampayo, 2002b) but *Mytilus* sp. usually contain only low amounts of DTX-3 (see *1.5.2*). In Swedish blue mussels, analysis of DTX-3 is currently not included in monitoring for DST, thus not much is known about the presence of DTX-3. Therefore, a preliminary study was performed where the content of DTX-3 was analysed in 20 random subsamples from the experiment in **paper IV**, using the application of PPIA for determination of DTX-3 in shellfish according to Mountfort *et al.* (2001) (see *2.3* and table 1). The proportion of acyl-OA to total toxin content was significant in the mussel tissue, ranging from 23 to 55% on

Table 1. Okadaic acid (OA eq., µg*g^{-1} digestive gland), acylated okadaic acid (OA-acyl, µg*g^{-1}) and percentage OA-acyl of total OA equivalents (% OA-acyl) in mussel digestive glands. Random subsamples from each temperature regime were analysed by protein phosphatase inhibition assay (PPIA) before and after hydrolysis. Values are means ± SD with number of replicates inside brackets.

Time (days)	Temperature (°C)	OA (µg*g^{-1})	OA-acyl (µg*g^{-1})	% OA-acyl
8	18	1.05±0.59 (4)	1.05±0.38 (4)	51±19
8	24	1.00±0.23 (3)	0.86±0.51 (3)	44±18
16	18	0.72±0.49 (3)	0.83±0.40 (3)	55±16
16	24	0.81±0.74 (4)	0.37±0.35 (4)	32±27
24	18	0.74±0.75 (4)	0.61±0.52 (4)	45±15
24	24	0.35±0.26 (3)	0.18±0.26 (3)	23±21

average. Thus, biotransformation could be an important step for elimination of OA in Swedish mussels. Future studies on depuration of DST in mussels should include the analysis of biotransformation products and estimates of conversion rates. The findings of large amounts of DTX-3 in Swedish blue mussels imply that they need to be accounted for in monitoring of DST since they may constitute an additional risk for consumers of mussels. Rapid hydrolysis of DTX-3 by lipases to form OA is likely to occur in the human stomach, increasing the initial toxicity of the mussel meat (Yasumoto, 2000).

3.3. Large-scale depuration

In **paper V**, the possibilities and efficiency of large-scale depuration for toxic mussels were explored by performing a field experiment. Mussels growing in a toxic environment in the outer archipelago were relocated to a secluded fjord north of the island of Orust on the Swedish west coast. In this fjord system, long-term monitoring for DST in local mussel populations has revealed consistently lower or non-detectable levels of toxins compared to outer areas (Haamer, 1995b; Lindahl & Andersson, 1996; Lindegarth, 1997), thus the fjord was considered a suitable area for depuration. The principle of the method was to keep the mussels in land-based tanks placed near the water using natural sea water for depuration. One of the advantages with this type of system is the possibility to move the tanks to areas where predicted water toxicity is low. Even greater flexibility could be achieved system if the tanks were installed on movable rafts. This would also allow that harvest and subsequent transportation to potential depuration areas could be performed at sea. Using high-capacity submerged pumps, sea water was pumped into the tanks with a bottom-up flow direction, supplying the mussels with oxygen and food particles.

Approximately 1000 kg of toxic mussels (600 µg OA*kg^{-1} mussel meat), cultured on a long-line farm, were harvested in late August, 2000, and immediately transported to the experimental site, 70-80 km south of the farm. Two tanks were installed for depuration, each containing 400-500 kg mussels. The depuration trial was conducted for five weeks with a weekly sampling frequency both at the farm and the fjord site. Sampling at the farm site was considered an important control for evaluation of the depuration efficiency in the fjord. Apart from weekly analysis of DST in mussels, we analysed the toxicity in plankton samples together with plankton identification and enumeration. Also, the importance of non-toxic food resources for depuration efficiency was evaluated Meat yield and dry weights of the mussels were used as measures of product quality.

Depuration of OA was successful during the first three weeks. Significantly lower

concentrations of OA in mussels at the fjord site were detected already after one week of depuration. OA concentration was further reduced after two and three weeks. No difference in mussel toxicity between tanks was detected which indicated that depuration was equally effective in both tanks. The rate of depuration was constant during these first three weeks which was evident from the high score for the correlation coefficient of the exponential function (r^2=0.99). Depuration half-life was calculated to 7 days. Thus, a 50% reduction was achieved already after 7 days which is a relatively fast rate for DST depuration (see *3.2*). Considering the safety regulation limit for DST in mussels (160 µg OA*kg^{-1}), it would have been possible to market these mussels after 12 days of depuration in the fjord without any substantial loss in product quality.

Interestingly, the rate of depuration of OA observed during the first three weeks was highly similar to what was found during the experiment in **paper IV** ($t_{1/2}$= 8 days). In **paper IV,** mussels from the same long-line farm as in **paper V** were used and the depuration experiment was performed only ten days prior to this field study. The physiological status and toxin history of mussels used in both **paper IV** and **V** can thus be considered similar and comparisons of depuration rates between these experiments are therefore relevant. More or less identical rates and depuration kinetics were found in these papers regardless of whether mussels were depurated in the lab under differing conditions or in the field using natural seawater (fig. 7).

In the farm, mussels remained toxic throughout the experiment except for the last sampling occasion where levels of OA were moderately reduced. Using data on several plankton variables, we could extract additional information about the potential causative factors determining the toxicity levels in the mussels. A preliminary analysis of the total abundance of phytoplankton as a measure of food availability showed no difference in this factor between locations during the experiment. Thus, higher amounts of food available for mussels in the fjord area did not explain the depuration success in the fjord compared to the farm. Instead, toxicity analysis of plankton samples confirmed the presence of DST in the water at all sampling occasions in the farm, suggesting that mussels continued to ingest toxins at the farm site. In the fjord, plankton extracts were non-toxic during the first period. The absence of toxins in the plankton community is the most probable cause for the reduction of OA in the experimental mussels. Although elimination of OA in mussels was fast and effective in the fjord, accumulation of DTX-1 occurred during the experiment. A slow increase in concentration of DTX-1 was initially observed but between the two last weeks of the experiment, DTX-1 rapidly increased to levels above the limit for consumption. The increase coincided with high levels of plankton toxicity detected in water samples from the fjord at week four and five. This pattern of DTX-1 accumulation was somewhat unexpected since the experimental site was selected because of its “low toxicity” history. It was concluded that this fjord system is not always safe to use for depuration purposes.

An interesting observation made in this study. DTX-1 was only detected in mussels at the experimental site and never in mussels from the farm. Plankton identification and counting together with concurrent analysis of toxicity in plankton extracts enabled identification of the causative species for DTX-1 production in the fjord. DTX-1 in the mussels was correlated to high plankton toxicity and numbers of *D. acuta*. The same species was also present in similar concentrations in the farm area but even so, this species did not appear to produce DTX-1 since this toxin was not found in mussels at the farm. Thus, different toxin profiles in *D. acuta* between these sites seem to exist which

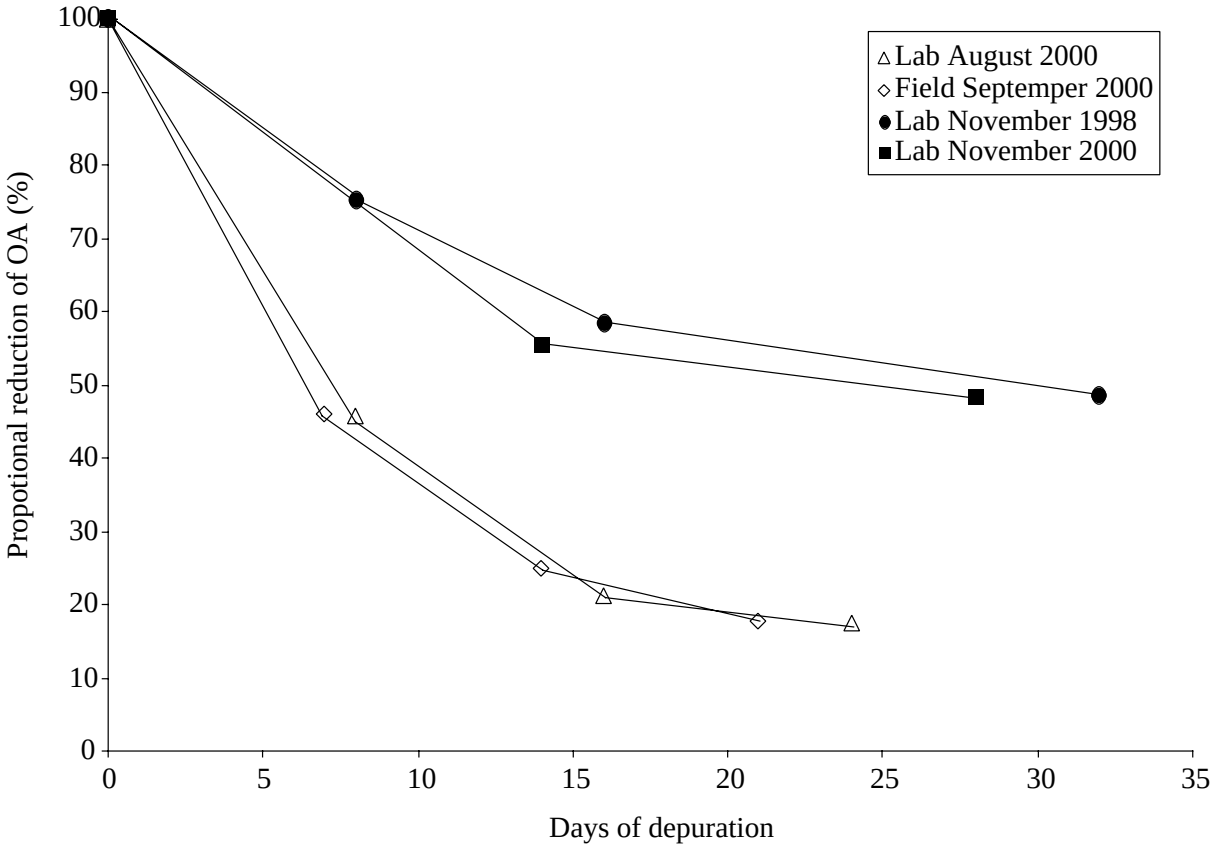


Fig. 7. A summary of four different depuration experiments performed 1998-2000 during different times of the year. The start value in each experiment was set to 100% and each consecutive value is then calculated as the percentage OA present in the tissue compared to the start value.

suggest that (1): the populations are genetically distinct (2): differences in environmental conditions induce variability in the production of DST compounds. A case of relatively high numbers of *D. acuta* in the fjord coupled to an inability to detect toxicity in the plankton samples suggested that the toxin content per cell varies in time. Variability in toxicity per cell was also confirmed in the farm site. No correlation between concentrations of *Dinophysis* and plankton toxicity could be seen during the whole period. Highly variable toxin contents per cell for both *D. acuta* and *D. acuminata* have also been observed in other parts of Europe (Andersen *et al.*, 1996; Aune *et al.*, 1996; Dahl & Johannessen, 2001; Fernández *et al.*, 2002; Marcaillou *et al.*, 2002). Consequently, the complexity in behaviour and ecophysiology of *Dinophysis* sp. indicate that cell counts of *Dinophysis* sp. alone are not a sufficient variable to use as an early warning for toxic events in shellfish. To improve the ability to predict toxicity in mussels, analysis of the factual toxin content in plankton samples should be included in monitoring programs (Godhe *et al.*, 2002).

To conclude, the current method using tanks for depuration of mussels is considered a promising option for mussel farmers. The crucial matter is to find locations where the probability for occurrence of toxin-producing *Dinophysis* sp. is low.

3.4. Depuration: summary and conclusions

A summary of the results from the depuration experiments in **paper III, IV** and **V** together with the results from the laboratory experiment where the effects of salinity and

temperature were tested (fig. 6) are illustrated in figure 7. For comparative reasons, the start value in each experiment was set to 100% and each consecutive value was then calculated as the percentage OA present in the tissue compared to the start value. Since no significant differences due to environmental conditions (treatments) were detected in the experimental works (**paper III, IV** and fig. 6), values represent the mean of all treatments included in each experiment. As evident from this summary, depuration kinetics were highly similar for the two laboratory experiments performed in November 1998 (**paper III**) and 2000 (fig. 6), even though the methodological approach and conditions used differed between these experimental occasions. For example, a static experimental setup was used in **paper III** whereas in the 2000 experiment, mussels were depurated in a flow-through system. The equal rates observed suggested that depuration was unaffected by the method used. Similarly, the average elimination rate of OA observed in the laboratory in August 2000 (**paper IV**) was more or less identical to that detected during the first three weeks in the large-scale field experiment in September, 2000 (**paper V**). This suggests that both field and laboratory conditions may be equally effective for depuration of mussels. This figure also illustrates the large rate differences between depuration performed in November compared to August and September and provides evidence for the arguments that the physiological status of the mussels (season) may regulate the elimination rate of OA.

3.5. Species-specific accumulation of DST

In **paper VI**, DST content in three bivalve species was compared in order to investigate species-specific differences in accumulation of these toxins. General knowledge about interspecific differences are important for the aquaculture and fisheries industries enabling efforts to be directed towards species with low toxicity during periods when other species contain elevated levels of algal toxins. As an example, the authorities in Maine, U.S., have enforced species-specific harvest and closure of fisheries regarding paralytic shellfish toxins (PST) in bivalves for many years (Dr. S. Shumway, pers. Comm.). Information about differential accumulation of toxins among various bivalve species can also provide guidelines as to the physiological processes that may be relevant to study during toxic events.

In Sweden, spatial and temporal variability in levels of OA and DTX-1 in blue mussels along the Swedish west coast has been documented since 1984 (see *1.4.3*). However, surprisingly little is known about the presence of DST in other commercial bivalve species such as the European oyster, *Ostrea edulis* and the edible cockle, *Cerastoderma edule*. At present, small-scale fisheries for oysters and cockles are conducted along the coast with a potential to increase the catch in the future. Oysters and cockles co-exist with blue mussels in shallow habitats and exploit the same food resource which suggest that they have the same possibility to accumulate DST when toxic *Dinophysis* sp. are present in the water column.

A puzzling observation was made during the summer of 1998. High levels of OA in farmed mussels were recorded in the North of Bohuslän whereas oysters and cockles sampled from the vicinity of this farm contained non-detectable levels of OA. Later that year, a field survey was designed to test the consistency of this observation. Mussels, oysters and cockles were simultaneously collected from two regions (100 km apart) during a time when elevated levels of OA in mussels had been detected for two months. Within each region, sampling was done at two locations (5 km apart) and in each location, individual specimens were collected within a 20 meters radius. Using a small-scale sampling strategy is an important part in studies on species-specific differences in

accumulation of toxins since it excludes the effects of differential exposure to the toxic algae. To our knowledge, this was the first paper where the accumulation of DST was compared among species from this aspect. This spatial experimental design also enabled us to evaluate the relevant scales for variability in concentrations of DST among species.

Applying PPIA for analysis of DST content in the bivalves, concentrations of DST above the regulation limit were detected in blue mussels from all locations (OA confirmed by HPLC as the principal DST). There were no significant differences in DST content between regions or locations within regions which suggested that the toxic event was evenly spread in mussels along the coast on this occasion. In contrast to mussels, levels of DST were below the detection limit in all oysters and cockles sampled in this study. This provided strong evidence for interspecific differences in behaviour and/or physiological response to the toxic algae or the assimilated toxin itself. Some possibilities which could explain this difference were discussed. Feeding inhibition in the presence of toxic algal species as well as pre- and/or post-ingestive selection in order to reduce the uptake of DST may be operating in oysters and cockles. If so, this suggests that these species are affected by the toxins which results in behavioural responses to the DST-producing algae. Effects on feeding and selective mechanisms have been observed in many bivalve species as a response to PST where deleterious effects due to these neurotoxic compounds are observed in the organism (see *1.2.5* and Bricelj & Shumway, 1998; Landsberg, 2002) but there are no previous studies showing selective behaviour against algae containing DST. Therefore, the second model discussed in **paper VI**, which suggest that biotransformation of DST occurs in oysters and cockles, is a more plausible explanation of observed results. As discussed in section *3.2*, there is convincing evidence that some bivalve species such as scallops, cockles, oysters and clams rapidly convert the DST parent compounds to their respective acylated form (DTX-3). Thus, these species may contain >95% DTX-3 of the total DST load (Suzuki *et al.*, 1998; Suzuki & Mitsuya, 2001; Vale & Sampayo, 2002a; Vale & Sampayo, 2002b). However, the acylated compounds are not detected using the traditional HPLC or PPIA methods as in **paper VI**. (see *2.2*). Although the aim of **paper VI** was not to study and analyse biotransformation products of DST, the findings indicate that this may be an important mechanism in Swedish oysters and cockles. Vale & Sampayo (1999) and Vale & Sampayo (2002a) have confirmed human poisoning incidences due to consumption of shellfish containing high proportions of acylated DST products. This again emphasizes the importance of including analysis of DTX-3 in monitoring programs. Also, recent evidence that bioconversion is such a widespread phenomena among various species underlines this as an important route for detoxification of DST in many bivalves (Vale & Sampayo, 2002b). As stated earlier, the exception is *Mytilus* sp., which usually contain only low proportions of DTX-3. Therefore, it seems likely that blue mussels employ other mechanisms to reduce the toxicity of OA and can therefore tolerate higher levels of unconverted OA than other species. Potential mechanisms have been proposed in previous sections of this thesis.

4. Concluding remarks

Although the questions regarding the specific physiological mechanisms involved in protection and depuration of OA in blue mussels remain partially unanswered, some general conclusions can be drawn from the work presented in this thesis. Mussels appear to be insensitive to the cytotoxic and secondary effects of OA. This would explain why mussels accumulate high levels of OA and nevertheless survive during extended periods of exposure in the field. In contrast to mussels, oysters and cockles do not accumulate OA to the same degree. This might suggest that these species are more sensitive to the harmful effects of this toxin. This interspecific difference in accumulation of OA could be exploited in the culture and fishery of bivalve molluscs. Depuration of OA in mussels has been achieved in all experiments. However, it has not been possible to accelerate the rate of depuration by manipulating the environmental conditions. The variability in depuration rates observed both in the field and the laboratory is likely to be correlated to seasonal changes in the physiological status of the mussel. These results have important implications for the development of depuration systems for mussels. The most cost-effective depuration of mussels could be achieved under conditions where mussels are self-depurated in sea water free of toxin-producing dinoflagellates, either in the field or the laboratory, with a predicted faster rate of elimination during warmer compared to colder seasons.

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6. References

Aguilera, A., González-Gil, S., Costas, E., López-Rodas, V., 1997. The biological function of okadaic acid in dinoflagellates: A specific mitogenic factor of *Prorocentrum lima* . Revista de Biología Marina y Oceanografía 32 (1), 37-44

Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman, M.M., 1999. Biochemical, cellular and pharmacological aspects of the multidrug transporter. Annu. Rev. Pharmacol. Toxicol. 39 361-398

Amzil, Z., Pouchus, Y.F., Le Boerff, J., Roussakis, C., Verbist, J.F., Marcaillou-Lebaut, C., Masselin, P., 1992. Short-time cytotoxicity of mussel extracts: a new bioassay for okadaic acid detection. Toxicon 30, 1419-1425

Andersen, P. 1996. Design and implementation of some harmful algal monitoring systems. IOC Technical Series no. 44. UNESCO, Paris. 102 pp.

Andersen, P., Hald, B., Emsholm, H., 1996. Toxicity of *Dinophysis acuminata* in Danish coastal waters. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 281-284

Aune, T., Yasumoto, T., Engeland, E., 1991. Light and scanning electron microscopic studies on effects of marine algal toxins toward freshly prepared hepatocytes. Journal of Toxicology and Environmental Helth 34, 1-9

Aune, T., Yndestad, M., 1993. Diarrhetic shellfish poisoning. In: Falconer, I. R., (Ed.), Algal toxins in seafood and drinking water. Academic Press, San Diego, pp. 87-104

Aune, T., Strand, Ö., Aase, B., Weidemann, J., Dahl, E., Hovgaard, P., 1996. The Sognefjord in Norway, a possible location for mussel farming? In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 73-76

Aune, T., 1997. Health effects associated with algal toxins from seafood. In: Seiler, J. P., Vilanova E., (Ed.), Applied toxicology. Approaches through basic science. Arch. Toxicol. 19, pp. 389-397

Aune, T., Sörby, T., Yasumoto, T., Ramstad, H., Landsverk, T., 2002. Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. Toxicon 40, 77-82

Bard, S. M., 2000. Multixenobiotic resistance as a cellular defence mechanism in aquatic organisms. Aquat. Tox. 48, 357-389

Bayne, B.L., Thompson, R.J., Widdows, J., 1973. Some effects of temperature and food on the rate of oxygen consumption by *Mytilus edulis* L. In: Wieser, W., (Ed.), Effects of temperature on ectothermic organisms. Springer-Verlag, Berlin, pp. 181-193

Bialojan, C., Takai, A., 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Biochem. J. 256, 283-290

Birmelin, C., Pipe, R.K., Goldfarb, P.S., Livingstone, D.R., 1999. Primary cell-culture of the digestive gland of the marine mussel *Mytilus edulis*: a time-course study of antioxidant- and biotransformation-enzyme activitiy and ultrastructural changes. Mar. Biol. 135, 65-75

Björk, M., Gilek, M., 1997. Bioaccumulation kinetics of PCB 31, 49 and 153 in the blue mussel, *Mytilus edulis* L. as a function of

algal food concentration. Aquat. Tox. 38, 101-123

Blanco, J., Fernández, M., Marino, J., Reguera, B., Míguez, A., Maneiro, J., Cacho, E., Martínez, A., 1995. From *Dinophysis* spp. toxicity to DSP outbreaks: a preliminary model of toxin accumulation in mussels. In: Lassus, P., Arzul G., Erard E., Gentien P., Marcaillou C., (Ed.), Harmful marine algal blooms. Lavoisier, Intercept Ldt, Paris, pp. 777-782

Blanco, J., Fernández, M.L., Míguez, A., Morono, A., 1999. Okadaic acid depuration in the mussel *Mytilus galloprovincialis*: one- and two-compartment models and the effect of environmental conditions. Mar. Ecol. Prog. Ser. 176, 153-163

Boe, R., Gjertsen, B.T., Vintermyr, O.K., Houge, G., Lanotte, M., Doskeland, S.O., 1991. The protein phosphatase inhibitor okadaic acid induces morfological changes typical of apoptosis in mammalian cells. Exp. Cell Res. 195, 237-246

Bricelj, M.V., Shumway, S.E., 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics and biotransformation. Reviews in Fisheries Science 6 (4), 315-383

Cajaraville, M.P., Abascal, I., Etxeberria, M., Marigomez, I., 1995. Lysosomes as cellular markers of environmental pollution: Time-and dose-dependent responses of the digestive lysosomal system of mussels after petroleum hydrocarbon exposure. Env. Toxicol. Wat. Qual. 10 (1), 1-8

Carmody, E.P., James, K.J., Kelly, S.S., 1996. Dinophysistoxin-2: The predominant diarrhoetic shellfish toxin in Ireland. Toxicon 34 (3), 351-359

Chambers, T.C., Raynor, R.L., Kuo, J.F., 1993. Multidrug-resistant human KB carcinoma cells are highly resistant to the protein phosphatase inhibitors okadaic acid and calyculin A. Analysis of potential mechanisms involved in toxin resistance. Int. J. Cancer 53, 323-327

Cohen, P., 1989. The structure and regulation of protein phosphatases. Annu. Rev. Biochem. 58, 453-508

Cohen, P., Cohen, P.T.W., 1989. Protein phosphatases come of age. J. Biol. Chem. 264 (36), 21435-21438

Cohen, P., Holmes, C.B.F., Tsukitani, Y., 1990. Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem. Sci. 15, 98-102

Cohen, P., 1994. The discovery of protein phosphatases: From chaos and confusion to an understanding of their role in cell regulation and human disease. BioEssays 16, (8), 583-588

Cook, P.A., Gabbot, P.A., 1978. Glycogen synthetase in the sea mussel *Mytilus edulis* L.- I. Purification, interconvention and kinetic properties of the I and D form. Comp. Biochem. Physiol. 60B, 419-421

Córdova, J.L., Bustamante, J., Cárdenas, L., 2001. Specific inhibition of endogenous shellfish protein phosphatase that could be used as a direct reporter of diarrhetic shellfish poison. J. Shellfish Res. 20 (1), 63-67

Cornwall, R., Holland Toomey, B., Bard, S., Bacon, C., Jarman, W.M., Epel, D., 1995. Characterization of multixenobiotic/multidrug transport in the gills of the mussel *Mytilus californianus* and identification of environmental substrates. Aquat. Tox. 31, 277-296

Croci, L., Toti, L., De Medici, D., Cozzi, L., 1994. Diarrhetic shellfish poisoning in mussels: comparison of methods of detection and determination of the effectiveness of depuration. J. Int. Food Microbiol. 24, 337-342

Dahl, E., Johannessen, T., 2001. Relationship between occurrence of *Dinophysis* species (Dinophyceae) and shellfish toxicity. Phycologia 40 (3), 223-227

Daranas, A.H., Norte, M., Fernández, J.J., 2001. Toxic marine microalgae. Toxicon 39, 1101-1132

De Zwaan, A., Mathieu, M., 1992. Cellular energy metabolism in the Mytilidae: an overview. In: Gosling, E., (Ed.), The mussel Mytilus: ecology, physiology, genetics and culture. Elsevier, Amsterdam, pp. 223-307

Della Loggia, R., Sidari, L., Honsell, G., Sosa, S., Viezzoli, A., Tubaro, A., 1998. The PP2A inhibition assay for monitoring: two years of experience. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 549-550

Draisci, R., Lucentini, L., Giannetti, L., Boria, P., Stacchini, A., 1995. Detection of diarrhoeic shellfish toxins in mussels from Italy by ionspray liquid chromatography-mass spectrometry. Toxicon 33 (12), 1591-1603

Edebo, L., Lange, S., Li, X.P., Allenmark, S., Lindgren, K., Thompson, R., 1988. Seasonal, geographic and individual variation of okadaic acid content in cultivated mussels in Sweden. APMIS 96, 1036-1042

Endicott, J.A., Ling, V., 1989. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu. Rev. Biochem. 58, 137-171

Farrington, J.W., 1989. Bioaccumulation of hydrophobic organic pollutant compounds. In: Levin, S. A., Harwell J. R., Kelly J. R., Kimball K. D., (Ed.), Ecotoxicology: Problems and Approaches. Springer-Verlag, New York, pp. 279-313

Fernández, M.L., Míguez, A., Cacho, E., Martinez, A., 1996a. Sanitary control of marine biotoxins in the European Union. National references laboratories network. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 11-14

Fernández, M.L., Míguez, A., Cacho, E., Martínez, A., 1996b. Detection of okadaic acid esters in the hexane extracts of Spanish mussels. Toxicon 34 (3), 381-387

Fernández, M.L., Míguez, A., Morono, A., Cacho, E., Martínez, A., Blanco, J., 1998. Detoxification of low polarity toxins (DTX-3) from mussels *Mytilus galloprovincialis* in Spain. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 449-452

Fernández, M.L., Reguera, B., Ramilo, I., Martínez, A., 2002. Toxin content of *Dinophysis acuminata* , *D. acuta* and *D. caudata* from the Galician rias bajas. In: Hallegraeff, G. M., Blackburn S. I., Bolch C. J., Lewis R. J., (Ed.), Harmful algal blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 360-363

Fernández-Sánchez, M.-T., García-Rodríguez, A., Díaz-Trelles, R., Novelli, A., 1998. Okadaic acid induces neuronal apoptosis. Role of calcium and trophic factors. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 577-580

Fladmark, K.E., Serres, M.H., Larsen, N.L., Yasumoto, T., Aune, T., Döskeland, S.O., 1998. Sensitive detection of apoptogenic toxins in suspension cultures of rat and salmon hepatocytes. Toxicon 36 (8), 1101-1114

Fontal, O.I., Leira, F., González, J.C., Vieyetes, M.R., Vieites, J.M., Botana, L.M., 1998. Determination of okadaic acid levels by HPLC and fluorescent microplate assay: a comparison of both methods. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 547-548

Fujiki, H., Suganuma, M., 1993. Tumor promotion by inhibitors of protein phosphatase 1 and 2A: the okadaic acid class of compounds. Adv. Canc. Res. 61, 143-194

Gabbot, P.A., 1986. Glycogen synthetase in the sea mussel *Mytilus edulis* L. -II. Seasonal changes in glycogen synthetase activity in the mantle tissue. Comp. Biochem. Physiol. 83 B, 197-207

Gago, A., Rodriguez-Vazquez, J.A., Leao, J.M., Comesana, M., Quilliam, M.A., Thibault, P., 1996. 1985-1995: A decade of studies on PSP and DSP toxins in Spanish mussels by chemical methods. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 7-10

Godhe, A., Svensson, S., Rehnstam-Holm, A.-S., 2002. Oceanographic settings explain fluctuations in *Dinophysis* spp. and concentrations of Diarrhetic Shellfish Toxin in the plankton community within a mussel farm area on the Swedish west coast. Mar. Ecol. Prog. Ser. 240, 71-83

Haamer, J., Andersson, P-O., Lange, S., Li, X.P., Edebo, L., 1990a. Effects of transplantation and reimmersion of mussels *Mytilus edulis* L. on their contents of okadaic acid. J. Shellfish Res. 9 (1), 109-112

Haamer, J., Andersson, P-O., Lindahl, O., Lange, S., Li, X.P., Edebo, L., 1990b. Geographic and seasonal variation of okadaic acid content in farmed mussels, *Mytilus edulis* L., along the Swedish west coast. J. Shellfish Res. 9 (1), 103-108

Haamer, J. 1995a. Phycotoxin and oceanographic studies in the development of the Swedish mussel farming industry. PhD Thesis, Dept. of Ocenanography and Clinical Bacteriology, Göteborg University, Göteborg

Haamer, J., 1995b. Presence of the phycotoxin okadaic acid in mussels (*Mytilus edulis*) in relation to nutrient composition in a Swedish coastal water. J. Shellfish Res. 14 (1), 209-216

Haamer, J., 1996. Improving water quality in a eutrophicated fjord system with mussel farming. Ambio 5, 356-362

Haamer, J., Rehnstam-Holm, A.-S., Edebo, L., Lindahl, O., Norén, F., Hernroth, B. 1999. Strategisk musselodling för att skapa kretslopp och balans i ekosystemet-kunskapsöversikt och förslag till åtgärder. Report no. 6 from Fiskeriverket, Göteborg, pp. 5-29 (In Swedish)

Haamer, J., Rodhe, J., 2000. Mussel, *Mytilus edulis* (L.) filtering of the Baltic sea outflow through the Öresund-an example of a natural, large-scale ecosystem restoration. J. Shellfish Res. 19 (1), 413-421

Hallegraeff, G.M., 1993. A rewiev of harmful algal blooms and their apparent global increase. Phycologia 32(2), 79-99

Hamano, Y., Kinoshita, Y., Yasumoto, T., 1986. Enteropathogenicity of diarrhetic shellfish toxins in intestinal models. J. Food Hyg. Soc. Jpn. 27, 375-379

Hawkins, A.J.S., Bayne, B.L., Clarke, K.R., 1983. Co-ordinated rythms of digestion, absorption and excretion in *Mytilus edulis* (Bivalvia: Mollusca). Mar. Biol. 74 (1), 41-48

Hawkins, A.J.S., Bayne, B.L., 1984. Seasonal variation in the balance between physiological mechanisms of feeding and digestion in *Mytilus edulis* (Bivalvia: Mollusca). Mar. Biol. 82, 233-240

Hawkins, A.J.S., Bayne, B.L., 1985. Seasonal variation in the relative utilization of carbon and nitrogen by the mussel *Mytilus edulis*: budgets, conversion efficiencies and maintenance requirements. Mar. Ecol. Prog. Ser. 25, 181-188

Hawkins, A.J.S., Bayne, B.L., 1992. Physiological interrelations, and the regulation of production. In: Gosling, E., (Ed.), The mussel *Mytilus*: ecology, physiology, genetics and culture. Elsevier, Amsterdam, pp. 171-222

Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P., Hardie, D.G., 1989. Effects of the tumour-promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 337, 78-81

Hernroth, B., Larsson, A., Edebo, L., 2000. Influence on uptake, distribution and elimination of *Salmonella typhimurium* in the blue mussel, *Mytilus edulis* , by cell surface properties of the bacteria. J. Shellfish Res. 19 (1), 167-174

Hickman, R. W., 1992. Mussel cultivation. In: Gosling, E., (Ed.), The mussel *Mytilus*: ecology, physiology, genetics and culture. Elsevier, Amsterdam, pp. 465-510

Higgins, C.F., 1992. ABC transporters: from microorganisms to man. Ann. Rev. Cell Biol. 8, 67-113

Holmes, C., Luu, H.A., Carrier, F., Schmitz, F., 1990. Inhibition of protein phosphatases-1 and -2A with acanthifolicin. Comparison with diarrhetic shellfish toxins and identification of a region on okadaic acid important for phosphatase inhibition. FEBS letter 270, 216-218

Honkanen, R.E., 1996. Detection of DSP-toxins, okadaic acid, and dinophysis toxin-1 in shellfish by serine/threonine protein phosphatase assay. Food Chem. Cont. 79 (6), 1336-1343

Honkanen, R.E., Stapleton, J.D., Bryan, D.E., Abercrombie, J., 1996. Development of a protein phosphatase-based assay for the detection of phosphatase inhibitors in crude whole cell and animal extracts. Toxicon 34 (11-12), 1385-1392

James, K.J., Bishop, A.G., Carmody, E.P., Kelly, S.S., 2000. Detection methods for okadaic acid and analogues. In: Botana, L. M., (Ed.), Seafood and freshwater toxins. Pharmacology, physiology and detection. Marcel Dekker, New York, pp. 217-238

James, K.J., Furey, A., Lehane, M., Ramstad, H., Aune, T., Hovgaard, P., Morris, S., Higman, W., Satake, M., Yasumoto, T., 2002. First evidence of an extensive northern European distribution of azaspiracid poisoning (AZP) toxins in shellfish. Toxicon 40 909-915

Jellet, J.F., 1993. Phytotoxins and shellfish aquaculture. World Aquaculture 24 (4), 32-43

Jörgensen, C.B., 1991. Bivalve filter feeding: hydrodynamics, bioenergetics, physiology and ecology, Olsen and Olsen (ed). Fredensborg, 140 pp

Karlsson, B., Rehnstam-Holm, A.-S. 2002. Algal toxins (DSP/PSP mussel infection events). Swedish National Report on Eutrophication Status in Kattegat and Skagerrak. OSPAR Assessment, pp. 42-44

Kat, M. 1983. *Dinophysis acuminata* blooms in the Dutch coastal area related to diarrhetic mussel poisoning in the Dutch Waddensea. Sarsia 68, 81-84

Kawamura, K.-I., Grabowski, D., Weizer, K., Bukowski, R., Ganapathi, R., 1996. Modulation of vinblastine cytotoxicity by dilantin (phenytoin) or the protein phosphatase inhibitor okadaic acid involves the potentiation of anti-mitotic effects and induction of apoptosis in human tumor cells. Brit. J. Cancer 73, 183-188

Kollberg, S. 1999. Beskrivning av den svenska musselnäringen. Vattenbrukarnas Riksförbund, Göteborg, 68 pp (In Swedish)

Krogh, P., Edler, L., Graneli, E., Nyman, U., 1985. Outbreak of Diarrhetic Shellfish Poisoning on the west coast of Sweden. In: Anderson, D. A., White, Baden D., (Ed.), Toxic Dinoflagellates. Elsevier Science Publishing, Amsterdam, pp. 501-503

Kumagai, M., Yanagi, T., Murata, M., Yasumoto, T., Kat, M., Lassus, P., Rodriguez-Vazquez, J.A., 1986. Okadaic acid as the causative toxin of diarrhetic shellfish poisoning in Europe. Agric.Biol.Chem. 50 (11), 2853-2857

Kurelec, B., Pivcevic', B., 1991. Evidence for a multixenobiotic resistance mechanism in the mussel *Mytilus galloprovincialis* . Aquat. Tox. 19, 291-302

Kurelec, B., 1995. Reversion of the multixenobiotic resistance mechanism in gills of a marine mussel *Mytilus galloprovincialis* by a model inhibitor and environmental modulators of P170-glycoprotein. Aquat. Tox. 33, 93-103

Köhler, A., Lauritzen, B., Jansen, D., Böttcher, P., Teguliwa, L., Krüner, G., Broeg, K., 1998. Detection of p-glycoprotein mediated MDR/MXR in *Carcinus maenas* hepatopancreas by immuno-gold-silver labeling. Mar. Env. Res. 48, 411-414

Laidley, C.W., Cohen, E., Casida, J.E., 1997. Protein phosphatase in neuroblastoma cells: ^{3}H-cantharidin binding site in relation to cytotoxicity. J. Pharmacol. Exp. Therapeutics 280 (3), 1152-1158

Landsberg, J.H., 1996. Neoplasia and biotoxins in bivalves: is there a connection? J. Shellfish Res. 15 (2), 203-230

Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. Rev. Fish. Sci. 10 (2), 113-390

Langton, R.W., 1977. Digestive rythms in the mussel *Mytilus edulis* L. Mar. Biol. 41, 53-58

Lawrence, J.E., Bauder, A.G., Quilliam, M.A., Cembella, A.D., 1998. *Prorocentrum lima* : a putative link to diarrhetic shellfish poisoning in Nova Scotia, Canada. In: Reguera, B., Blanco J., Fernandez M. L., Wyatt T., (Ed.), Harmful algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 78-79

Lee, J.S., Yanagi, T., Kenma, R., Yasumoto, T., 1987. Fluorometric determination of diarrhetic shellfish toxins by high pressure liquid chromatography. Agr. Biol. Chem. 51, 877-881

Lee, J.S., Igarashi, T., Fraga, S., Dahl, E., Hovgaard, P., Yasumoto, T., 1989. Determination of diarrhetic shellfish toxins in various dinoflagellate species. J. Appl. Phycol. 1, 147-152

Lindahl, O., Andersson, B., 1996. Environmental factors regulating the occurrence of *Dinophysis* spp. in the Koljö fjord, Sweden. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 269-272

Lindahl, O., Hageltorn, M. 1986. Detoxification experiments of DSP in blue mussels. 15th Nordic Veterinary Congress. 463-466

Lindegarth, M. 1997. Sammanställning och tolkning av de kontinuerliga mätningarna av okadasyrahalter längs den svenska västkusten. Sammanställning och tolkning av de kontinuerliga mätningarna av okadasyrahalter längs den svenska västkusten. Länsstyrelsen Västra Götaland, 17 pp. (In Swedish)

Livingstone, D.R., Lips, F., Garcia Martinez, P., Pipe, R.K., 1992. Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. Mar. Biol. 112, 265-276

Livingstone, D.R., Pipe, R.K., 1992. Mussels and environmental contaminants: molecular and cellular aspects. In: Gosling, E., (Ed.), The mussel *Mytilus*: ecology, physiology, genetics and culture. Elsevier Science Publishers, Amsterdam, pp. 425-464

Loo, L.-O., Rosenberg, R. 1983. Settling of *Mytilus edulis* at some localities on the Swedish west coast in 1981 and 1982. Havsfiskelaboratoriet, Lysekil, 14 pp

Lowe, D.M., Fossato, V.U., Depledge, M.H., 1995. Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an *in vitro* study. Mar. Ecol. Prog. Ser. 129, 189-196

MacKenzie, L., Holland, P., McNabb, P., Beuzenberg, V., Selwood, A., Suzuki, T., 2002. Complex toxin profiles in phytoplankton and greenshell mussels (*Perna canaliculus)*, revealed by LC-MS/MS analysis. Toxicon 40 (9), 1321-1330

Magnusson, K., Ekelund, R., Ingebrigtsen, K., Granmo, Å., Brandt, I., 2000. Tissue disposition of benzo[*a*]pyrene in blue mussel (*Mytilus edulis*) and effect of algal concentration on metabolism and depuration. Envir. Tox. Chem. 19 (11), 2683-2690

Marcaillou-Le Baut, C., Bardin, B., Bardouil, M., Bohec, M., Le Dean, L., Masselin, P., Truquet, P., 1993. DSP depuration rates of mussels reared in a laboratory and an aquaculture pond. In: Smayda, T. J., Shimizu Y., (Ed.), Toxic phytoplankton blooms in the sea. Elsevier Science Publisher, pp. 531-535

Marcaillou, C., Gentien, P., Lunven, M., Le Grand, J., Mondeguer, F., Danilou, M.M., Crassous, M.P. et al., 2002. *Dinophysis acuminata* distribution and specific toxin content in relation to mussel contamination. In: Hallegraeff, G. M., Blackburn S. I., Bolch C. J., Lewis R. J., (Ed.), Harmful algal blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 356-359

Marr, J.C., Hu, T., Pleasance, S., Quilliam, M.A., Wright, J.L.C., 1992. Detection of new 7-*O* -acyl derivatives of diarrhetic shellfish poisoning toxins by liquid chromatography-mass spectrometry. Toxicon 30 (12), 1621-1630

Minier, C., Akcha, F., Galgani, F., 1993. P-glycoprotein expression in *Crassostrea Gigas* and *Mytilus edulis* in polluted seawater. Comp. Biochem. Physiol. 106B (4), 1029-1036

Minier, C., Moore, M.N., 1996. Induction of multixenobiotic resistance in mussel blood cells. Mar. Env. Res. 42 (1-4), 389-392

Moore, M.N., Livingstone, D.R., Widdows, J., 1989. Hydrocarbons in marine molluscs: biological effects and ecological consequences. In: Varanasi, V., (Ed.), Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. Boca Raton, Florida, pp. 291-328

Moore, M.N., 1990. Lysosomal cytochemistry in marine environmental monitoring. Histochem. J. 22, 187-191

Moore, M.N., Willows, R.I., 1998. A model for cellular uptake and intracellular behaviour of particulate-bound micropollutants. Mar. Env. Res. 46 (1-5), 509-514

Morono, A., Fernández, L.M., Franco, J.M., Martínez, A., Reyero, I.M., Míguez, A., Cacho, E., Blanco, J., 1998. PSP and DSP kinetics in mussel, *Mytilus galloprovincialis*: effect of environmental parameters and body weight. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 445-448

Morton, B., 1983. Feeding and digestion in bivalvia. In: Saleuddin, A. S. M., Wilbur K. M., (Ed.), The mollusca. Physiology part 2. Academic Press, New York, pp. 65-148

Mountfort, D.O., Kennedy, G., Garthwaite, I., Quilliam, M., Truman, P., Hannah, D.J., 1999. Evaluation of the fluorometric protein phosphatase inhibition assay in the determination of okadaic acid in mussels. Toxicon 37, 909-922

Mountfort, D.O., Suzuki, T., Truman, P., 2001. Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. Toxicon 39, 383-390

Murakami, Y., Oshima, Y., Yasumoto, T., 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima* . Bull. Jpn. Soc. Sci. Fish. 48, 69-72

Murata, M., Shimatani, M., Sugitani, H., Oshima, Y., Yasumoto, T., 1982. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. Bull. Japan Soc. Sci. Fish. 48, 549-552

Newell, C.R., Shumway, S.E., Cucci, T.L., Selvin, R., 1989. The effects of natural seston particle size and type on feeding rates, feeding selectivity and food resource availability for the mussel *Mytilus edulis* L. 1758 at bottom culture sites in Maine. J. Shellfish Res. 8, 187-196

Nishiwaki, S., Fujiki, H., Suganuma, M., Furuyasuguri, H., Matsushima, R., Iida, Y., Ojika, M., Yamada, K., Uemura, D., Yasumoto, T., Schmitz, F. J., Sugimura, T., 1990. Structure-activity relationship with a series of okadaic acid derivatives. Carcinogenesis 11, 1837-1841

Owen, G., 1972. Lysosomes, peroxisomes and bivalves. Sci. Prog. Oxf. 60, 299-318

Peng, Y. G., Taylor, T. B., Finch, R. E., Switzer, R. C., Ramsdell, J. S., 1994. Neuroexicatory and neurotoxic actions of the amnesic shellfish poison, domoic acid. Neuroreport 5, 981-985

Phillips, D. J. H., 1993. Bioaccumulation. In: Calow, P., (Ed.), Handbook of ecotoxicology. Blackwell Scientific Publications, pp. 378-398

Pillet, S., Pereira, A., Braekman, J.-C., Houvenaghel, G., 1995. Patterns in long term accumulation of okadaic acid and DTX1 in blue mussels, *Mytilus edulis*, experimentally fed with the DSP-containing alga *Prorocentrum lima* . In: Lassus, P., (Ed.), Harmful algal blooms. Elsevier, Amsterdam, pp. 487-492

Poletti, R., Viviani, R., Casadei, C., Lucentini, L., Funari, E., Draisci, R., 1996. Decontamination dynamics of mussels naturally contaminated with diarrhetic toxins relocated to a basin of the Adriatic sea. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 429-432

Poli, M.A., Mende, T.J., Baden, D.G., 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. Mol. Pharmacol. 30, 129-135

Pugazhenthi, S., Yu, B., Gali, R.R., Khandelwal, R.L., 1993. Differential effects of calyculin A and okadaic acid on the glucose-induced regulation of glykogen synthase and phosphorylase activities in cultured hepatocytes. Bioch. et Biophys. Acta 1179, 271-276

Quilliam, M.A., 1995. Analysis of Diarrhetic Shellfish Poisoning Toxins in shellfish tissue by liquid chromatography with fluorometric and mass spectrometric detection. Journal of AOAC International 78 (2), 555-570

Ramstad, H., Larsen, S., Aune, T., 2001. The repeatability of two HPLC methods and the PP2A assay in the quantification of diarrhetic toxins in blue mussels (*Mytilus edulis*). Toxicon 39, 515-522

Reid, R. G. B., 1965. The structure and function of the stomach in bivalve molluscs. J. Zool. 147, 156-184

Ritz, V., Marwitz, J., Richter, E., Ziemann, C., Quentin, I., Steinfelder, H.J., 1997. Characterization of two pituitary GH3 cell sublines partially resistant to apoptosis induction by okadaic acid. Biochem. Pharmacol. 54, 967-971

Roach, P.J., 1981. Glykogen synthase and glykogen synthase kinase. Curr. Top. Cell. Reg. 20, 45-103

Sampayo, M.A., Alvito, P., Franca, S., Sousa, I., 1990. *Dinophysis* spp. toxicity and relation to accompanying species. In: Granéli, E., Sundström B., Edler L., Anderson D. M. M., (Ed.), Toxic marine phytoplankton. Elsevier, New York, pp. 215-220

Schmidt, K.N., Traenckner, E.B., Meier, B., Baeuerle, P.A., 1995. Induction of oxidative stress by okadaic acid is

required for activation of transcription factor NF-kB. J. Biol. Chem. 270 (45), 27136-27142

Seed, 1969. The ecology of *Mytilus edulis* L. (Lamellibranchiata) on exposed rocky shores. I. Breeding and settlement. Oecol. 3, 277-316

Seed, R., Suchanek, T.H., 1992. Population and community ecology of *Mytilus* . In: Gosling, E., (Ed.), The mussel Mytilus: ecology, physiology, genetics and culture. Elsevier, Amsterdam, pp. 87-169

Shimizu, Y., Kinoshita, M., Ooi, F., 1998. Highly sensitive, non-radioactive assay for protein phosphatase 1 and protein phosphatase 2A. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 537-540

Shumway, S.E., 1990. A review of the effects of algal blooms on shellfish and aquaculture. J. World Aquaculture Soc. 21 (2), 65-104

Silvert, W.L., Cembella, A.D., 1995. Dynamic modelling of phycotoxin kinetics in the blue mussel, *Mytilus edulis* , with implications for other marine invertebrates. Can. J. Fish. Aquat. Sci. 52, 521-531

Smayda, T.J., 1990. Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: Granéli, E., Sundström B., Edler L., Anderson D. M., (Ed.), Toxic Marine Phytoplankton. Elsevier Science Publishing Co., New York, pp. 29-40

Spacie, A., Hamelink, J.L., 1985. Bioaccumulation. In: (Ed.), Fundaments of aquatic toxicology. Hemisphere Publ. Corp., New York, pp. 495-525

Sueoka, E., Fujiki, H., 1998. Carcinogenesis of okadaic acid class tumor promoters derived from marine natural products. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 573-576

Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., 1988. Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. Proc. Nat. Acad. Sci. 85, 1768-1771

Suzuki, T., Yoshizawa, R., Kawamura, T., Yamasaki, M., 1996. Interference of free fatty acids from the hepatopancreas of mussels with the mouse bioassay for shellfish toxins. Lipids 31 (6), 641-645

Suzuki, T., Ota, H., Yamasaki, M., 1998. Direct evidence of transformation of Dinophysistoxin-1 to 7-O-Acyl-Dinophysistoxin-1 (Dinophysistoxin-3) in the scallop *Pactinopecten yessoensis* . Toxicon 37 (1), 187-198

Suzuki, T., Mitsuya, T., 2001. Comparison of dinophysistoxin-1 and esterified dinophysistoxin-1 (dinophysistoxin-3) content in the scallop *Pactinopecten yessoensis* and the mussel *Mytilus galloprovincialis*. Toxicon 39, 905-908

Svärdh, L. 2003. Histological studies of *Mytilus edulis* L. PhD Thesis, Dept. Marine Ecology, Göteborg University, Göteborg

Tachibana, K., Scheuer, P.J., Tsukitani, Y., Kikuchi, H., Van Eugen, D., Clardy, J., Gopichand, Y., Schmitz, F.J., 1981. Okadaic acid, a cytotoxic polyether toxin from two marine sponges of the genus *Halichondria*. J. Am. Chem. Soc. 103, 2469-2471

Takai, A., Murata, M., Torigoe, K., Isobe, M., Mieskes, G., Yasumoto, T., 1992. Inhibitory effects of okadaic acid derivatives on protein phosphatases. A study on structure-affinity relationship. Biochem. J. 284, 539-544

Tohda, H., Yasui, A., Yasumoto, T., Nakayasu, M., Shima, H., Nagao, M., Sugimura, T., 1994. Chinese Hamster ovary cells resistant to okadaic acid express a multidrug resistant phenotype.

Biochem. Biophys. Res. Comm. 203 (2), 1210-1216

Tripuraneni, J., Koutsouris, A., Pestic, L., de Lanerolle, P., Hecht, G., 1997. The toxin of diarrhetic shellfish poisoning, okadaic acid, increases intestinal epithelial paracellular permeability. Gastroenterology 112, 100-108

Tubaro, A., Florio, C., Luxich, E., Sosa, S., Della Loggia, R., Yasumoto, T., 1996a. A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. Toxicon 34 (7), 743-752

Tubaro, A., Florio, C., Luxich, E., Vertua, R., Della Loggia, R., Yasumoto, T., 1996b. Suitability of the MTT-based cytotoxicity assay to detect okadaic acid contamination of mussels. Toxicon 34 (9), 965-974

Underdal, B., Skulberg, O. M., Dahl, E., Aune, T., 1989. Disastrous bloom of *Chrysochromulina polylepis* (prymnesiophyceae) in Norwegian coastal waters 1988- mortality in marine biota. Ambio 18 (5), 265-270

Underdal, B., Yndestad, M., Aune, T., 1985. DSP intoxication in Norway and Sweden, autumn 1984-spring 1985. In: Anderson, W. a. B., (Ed.), Toxic Dinoflagellates. Elsevier Science Publishing, Amsterdam, pp. 489-494

Vale, P., Sampayo, M. A. M., 1999. Esters of okadaic acid and dinophysistoxin-2 in Portugese bivalves related to human poisonings. Toxicon 37, 1109-1121

Vale, P., Sampayo, M. A., 2002a. First confirmation of human diarrhoeic poisonings by okadaic acid esters after ingestion of razor clams (*Solen marginatus*) and green crabs (*Carcinus maenas*) in Aveiro lagoon, Portugal and detection of okadaic acid esters in phytoplankton. Toxicon 40, 989-996

Vale, P., Sampayo, M. A. M., 2002b. Esterification of DSP toxins by Portugese bivalves from the Northwest coast determined by LC-MS-a widespread phenomenon. Toxicon 40, 33-42

Van Dolah, F. M., 2000a. Diversity of marine and freshwater algal toxins. In: Botana, L. M., (Ed.), Seafood and freshwater toxins. Pharmacology, physiology and detection. Marcel Dekker, New York, pp. 19-43

Van Dolah, F. M., 2000b. Marine algal toxins: origins, health effects, and their increased occurrence. Environ. Health Perspect. 108, 133-141

Vieites, J. M., Leira Sanmartin, F., 2000. Incidence of marine toxins on industrial activity. In: Botana, L. M., (Ed.), Seafood and freshwater toxins. Pharmacology, physiology and detection. Marcel Dekker, New York, pp. 741-760

Vieytes, M. R., Fontal, O. I., Leira, F., Baptista de Sousa, J. M. V., Botana, L. M., 1997. A fluorescent microplate assay for diarrheic shellfish toxins. Anal. Biochem. 248, 258-264

Vieytes, M. R., Louzao, M. C., Alfonso, A., Cabado, A. G., Botana, L. M., 2000. Mechanism of action and toxicology. In: Botana, L. M., (Ed.), Seafood and freshwater toxins. Pharmacology, physiology and detection. Marcel Dekker, New York, pp. 239-256

Wang, S.J., Scavetta, R., Lenz, H.J., Danenberg, K., Danenberg, P.V., Schonthal, A.H., 1995. Gene amplification and multidrug resistance induced by the phosphatase-inhibitory tumor promoter, okadaic acid. Carcinogenesis 16, 637-641

Ward, J.E., Levinton, J.S., Shumway, S.E., Cucci, T., 1998. Particle sorting in bivalves: in vivo determination of the pallial organs of selection. Mar. Biol. 131, 283-292

Wera, S., Hemmings, B.A., 1995. Serine/threonine protein phosphatases. Biochem. J. 311, 17-29

Whittle, M.A., Gabbot, P.A., 1986. Glycogen synthase in the sea mussle *Mytilus edulis* L. III. Regulation by glucose in a mantle

tissue slice preparation. Comp. Biochem. Physiol. 83B, 209-214

Widdows, J., 1973. Effects of temperature and food on the heart beat, ventilation rate and oxygen uptake of *Mytilus edulis*. Mar. Biol. 20, 269-276

Widdows, J., Donkin, P., 1992. Mussels and environmental contaminants: bioaccumulation and physiological aspects. In: Gosling, E., (Ed.), The mussel *Mytilus*: ecology, physiology, genetics and culture. Elsevier Science Publishers, Amsterdam, pp. 383-425

Wieser, W., 1973. Temperature relations of ectotherms: a speculative view. In: Wieser, W., (Ed.), Effects of temperature on ectothermic organisms. Springer-Verlag, Berlin, pp. 1-24

Windust, A.J., Wright, J.L.C., McLachlan, J.L., 1996. The effects of the diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin-1, on the growth of microalgae. Marine Biology (Berlin) 126 (1), 19-25

Wright, J.L.C., Boyd, R.K., Defrietas, A.S.W., Falk, M., Foxall, R.A., Jamieson, W.D., Baycock, M.V., McCulloch, A.W., McInnes, A.G., Odense, P., *et al*, 1989. Identification of domoic acid, a neuroexicatory amino acid, in toxic mussels from eastern P. E. I. Can. J. Chem. 67, 481-490

Wright, J.L.C., Cembella, A.D., 1998. Ecophysiology and biosynthesis of polyether marine biotoxins. In: Anderson, D. M., Cembella A. D., Hallegraeff G.M., (Ed.), Physiological ecology of harmful algal blooms. Springer-Verlag, Heidelberg, pp. 427-451

Yanagi, T., Murata, M., Torigoe, K., Yasumoto, T., 1989. Biological activities of semisynthetic analogs of Dinophysistoxin-3, the major diarrhetic shellfish toxin. Agric. Biol. Chem. 53 (2), 525-529

Yasumoto, T., Oshima, Y., Yamaguchi, M., 1978. Occurence of a new type of shellfish poisoning in the Tohoku district. Bull. Jap. Soc. Sci. Fish. 44 (11), 1249-1255

Yasumoto, T., Oshima, Y., Sugawara, W., Fukuyo, Y., Oguri, H., Igarashi, T., Fujita, N., 1980. Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. Bull. Jpn. Soc. Sci. Fish. 46 1405-1411

Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G.K., Clardy, J., 1985. Diarrhetic shellfish toxins. Tetrahedron 41, 1019-1025

Yasumoto, T., Murata, M., Lee, J. S., Torigoe, K., 1989. Polyether toxins produced by dinoflagellates. In: Natori, S., Hashimoto K., Ueno Y., (Ed.), Mycotoxins and phycotoxins ´88. Elsevier, Amsterdam, pp. 375-382

Yasumoto, T., Fukui, M., Sakaksi, K., Sugiyama, K., 1995. Determinations of marine toxins in foods. J. AOAC Int. 78, 574-582

Yasumoto, T., 2000. Historic considerations regarding seafood safety. In: Botana, L. M., (Ed.), Seafood and freshwater toxins. Pharmacology, physiology and detection. Marcel Dekker, New York, pp. 1-17

Yusa, K., Tsuruo, T., 1989. Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. Cancer Res. 49, 5002-5006

I

EFFECTS OF OKADAIC ACID ON PROTEIN PHOSPHATASE AND GLYCOGEN SYNTHASE ACTIVITIES IN BLUE MUSSEL, *MYTILUS EDULIS*, AND RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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ABSTRACT

The effects of okadaic acid (OA) on protein phosphatase (PP) and glycogen synthase (GS) activity were investigated in blue mussel and rainbow trout. In both species, PP activity was inhibited by 200 nM I-2 as well as 2 nM OA, suggesting the presence of both type 1 and type 2A phosphatases. This shows that OA may disturb the phosphorylation state of enzymes in these species. GS activity was measured by an *in vitro* slice method; 10 µM OA inhibited GS activity in rainbow trout but not in blue mussel. Also, feeding mussels the OA-producer *Prorocentrum lima* had no effect on GS. These results indicate that there may be protective mechanisms against harmful effects of OA in blue mussel.

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) was reported in Sweden in 1984 in people who had consumed farmed blue mussels, *Mytilus edulis* [1]. The DSP outbreak was correlated with the occurrence of *Dinophysis* spp. in the water [2], and okadaic acid (OA) was identified as the main causative agent of DSP in Scandinavian shellfish [3]. High levels of OA are found in blue mussels for several months every year (unpublished data) and pose a severe threat to harvesters and consumers of these organisms.

The impact of OA on mammalian cells has been studied extensively. OA specifically inhibits protein phosphatase 1 (PP1) and 2A (PP2A) and causes general disturbances in reversible phosphorylation of enzymes [4, 5]. Depending on the dose and exposure time to OA, this inhibition generates effects such as increased glucose output and lipolysis [5], tumor promotion [6] and elevated levels of oxygen radicals [7] which may be harmful. PP1 and PP2A activity has also been studied in organisms as diverse as yeast [8], higher plants [9] and the OA-producer *Prorocentrum lima* [10]. PP1 and PP2A are products of the same gene family and it has been shown that it is a structurally very conserved group of enzymes [11]. OA is nowadays used as a tool to distinguish between different phosphatase activities in tissue [12].

So far, there is no documentation of the intracellular localization or effects of OA in shellfish naturally exposed to this compound. However, the behaviour and health status of shellfish during periods of OA accumulation have been studied. Laboratory feeding experiments have shown that shellfish accumulate OA, grow and survive without increased mortality on uni-algal diets of OA-producing species [13, 14]. In [13], clearance rate was similar between scallops fed with *P. lima* and the non-toxic diatom *Thalassiosira weissflogii*. In Swedish mussel populations, no apparent increased mortalities have been observed during blooms of toxic algae. These results indicate that shellfish remain unaffected when exposed to OA-producers. It is possible that these organisms have mechanisms to avoid harmful effects of OA.

In the present study, the intracellular effects of OA was investigated in *M. edulis* and cultured rainbow trout, *Onchorynchus mykiss* by measuring protein phosphatase (PP) and glycogen synthase (GS) activity. GS is a key enzyme in glycogen synthesis and its activity is regulated by reversible phosphorylation where the dephosphorylated form is active *in vivo*. GS has been identified in mussel tissue [15] and several authors have demonstrated the inhibition of GS activity by the action of OA on PP1 and PP2A in mammals [5, 16]. GS activity was studied in two different systems: an *in vitro* tissue slice method and an *in vivo* mussel feeding experiment.

MATERIALS AND METHODS

Materials

Potassium salt of OA (from Sigma Chemicals) was dissolved in 70 % ethanol; uridine-diphospho-glucose (UDPG) and glucose-6-phosphate (G-6-P) were from Boerhinger Mannheim; UDP-[^{14}C]-glucose (> 200 mCi/mmol) and [^{32}P]-ATP (>5000 Ci/mmol) were from Amersham; human recombinant inhibitor-2 (I-2) was from Upstate Biotechnology.

Animals

Blue mussels (6-7 cm length), and cultured juvenile rainbow trout (100-150 g), were kept in 10° C in recirculated artificial salt water before being used for the *in vitro* experiments. For the feeding experiment, mussels were conditioned during 4 weeks by giving them daily rations of *Isochrysis galbani* and *Tetraselmis suecica*.

In vitro experiment

The experiments were performed with modifications according to [17]. Digestive glands of mussels were dissected, the crystalline styles removed, and the glands placed in ice-cold artificial saline buffer with 1mM glucose. pH was adjusted to 7.3 by gassing with 97% O_2 + 3% CO_2. This buffer and gas was used as incubation medium during the experiments with mussel digestive gland tissue. Rainbow trout livers were dissected and placed in ice-cold Ringer solution (140 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM $MgSO_4$, 15 mM $NaHCO_3$, 1 mM KH_2PO_4, 5 mM Hepes) including 1 mM glucose. The Ringer was gassed with 0.3% CO_2 in air and pH adjusted to 7.8. The trout liver tissue was incubated in Ringer and gassed with the mixture during the experiments. Thin slices (0.1 g) of

Harmful Algae
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Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO 1998

cross-sectioned digestive gland and trout liver were placed in replicate test tubes containing 1 ml of medium which was gassed continously during the experiment. To the test tubes, OA was added and the tissue slices were incubated 2-6 hours. The incubation was terminated by homogenizing the tissue (see below). All experiments were done at 10° C.

Mussel feeding experiment

The experiment was performed during 8 days in August, 1996. Uni-algal cultures of the OA-producer *P. lima* (CCAP 1136/11) and the non-toxic *P. micans* (CCAP 1136/1) were grown in f/2 medium in a 16:8 L:D photocycle at 20° C. The algae cultures were measured for OA content before and after the experiment. Mussels (15 mussels/aquarium, 6 aquaria/treatment) were kept at 18° C in vigorously aereated aquaria each containing 5 l of filtered sea water. Treatment consisted of adding doses of *P. lima* and *P. micans* respectively to the aquaria at initial concentrations of 10^5 cells/liter, 6 times/day for 8 days. Replicate samples were removed after 2, 4 and 8 days. The digestive glands were dissected and frozen in liquid nitrogen for subsequent measurement of OA, PP and GS activity. Mussels removed were replaced by other marked individuals of the same size.

Okadaic acid analysis and protein phosphatase activity assay

The analysis of OA in the mussels and algae was done by the HPLC-method according to [18] modified by [19]. PP activity was measured according to [20] using a phosphatase assay kit. OA and I-2 was included in the assay to quantify PP1 and PP2A respectively in the tissue, 2 nM OA selectively inhibits PP2A activity whereas 200 nM I-2 inhibits PP1 activity [21]. Extracts were prepared by homogenization in 3 vol. of buffer (4 mM EDTA, 250 mM sucrose, 0.1 % 2-mercaptoethanol, 0.1 mM PMSF pH 7.0) and centrifuged at 16.000g for 15 min. The supernatant was frozen in liquid nitrogen and stored at -70° C. The homogenates were diluted to give an activity of 0.1 mU/ml before adding the inhibitors. One U is the amount of protein phosphatase that catalyze the dephosphorylation of 1 µmol substrate in one minute. Phosphatase activity is expressed as pmol/min/mg protein.

Glycogen synthase and protein assays

GS activity measurements were done with minor modifications according to [22]. Tissue was homogenized in 5 vol. of ice-cold buffer (50 mM Tris-HCl pH 7.8, 10mM EDTA, 100 mM KF, 0.5 mM PMFS, 1 mM DTT) and centrifuged at 16.000g, 15 min, 4° C. The supernatant was decanted, the pellet re-homogenized and centrifuged as above, and the second supernatant pooled with the first. The samples were frozen in liquid nitrogen and stored at -70° C until use. Thirty µl of tissue extract was added to 60 µl of a reaction mix (50 mM Tris-HCl pH 7.8, 25 mM KF, 20 mM EDTA, 1% glycogen, 6.7 mM UDPG, 0.05 µCi UDP-[^{14}C]-glucose). GS activity was measured in the absence (active form) and presence (total enzyme activity) of 10 mM G-6-P. After 30 min. of incubation at 30° C, 75 µl of the reaction mixture was spotted onto 2x2 cm chromatography paper, dropped into 66% ice-cold ethanol and washed in five changes of ethanol. The paper squares were put in 5 ml liquid scintillation cocktail and counted. Enzyme activity is calculated as nmol/mg prot./min. and the ratio between the active form and total activity is used to describe GS activity. Protein was estimated according to [23].

Statistical analyses

All analyses were done using one factor or two factor fixed effects ANOVA applying SNK aposteriori test for differences among means. Significances ($p<0.05$) are marked with (*). Values are presented as mean ± S.E.

RESULTS

Protein phosphatase activity

Mussel homogenates (15 mg protein/ml extract) were diluted 200-fold and rainbow trout tissue (20 mg protein/ml extract) 2000-fold to get 0.1 mU activity. Digestive gland tissue from non-toxic (no OA) and toxic mussel tissue (1.7 µg OA/g tissue) from the mussel feeding experiment was measured for PP activity. In non-toxic tissue, 48% of the total PP activity was measured in the presence of 200 nM I-2 and 50% when 2 nM OA was included (Table 1). In the presence of both inhibitors, a rest activity of 19 % was measured. In toxic mussel tissue, 48% of total PP activity was measured in the presence of 200 nM I-2 as in non-toxic mussels. However, the addition of 2 nM OA did not inhibit PP activity in the same amount; 83 % of total PP activity was still left when 2 nM OA was included and the addition of both inhibitors only reduced activity to 35 % compared to 19% in non-toxic mussel tissue. Also, the control value of toxic mussel tissue was significantly lower than control value from non-toxic mussels. In rainbow trout liver tissue, 37% and 62% of total activity was measured in the presence of 200 nM I-2 and 2 nM OA respectively. (Table 1). A rest activity of 22 % remained when both inhibitors were included in the assay.

Glycogen synthase activity

In the *in vitro* experiment, GS activity was initially measured at 0, 2, 4 and 6 hours of incubation in the absence of OA in both mussel and rainbow trout tissue (data not shown). The results showed that the presence of 1 mM glucose in the medium increased GS activity at 4 hours of incubation in both mussel and rainbow trout tissue and this effect of glucose remained at 6 hours in rainbow trout but not in mussel tissue. The increase of GS activity was due to the conversion of the inactive form of GS to the active in both organisms; GS activity was consistently higher (about 10 times) in trout liver slices compared to mussel tissue. Since the highest activity of GS was detected at 4 hours of incubation in mussel tissue and 6 hours in rainbow trout tissue, these incubation times were chosen to study the effects of OA.

Mussel slices were incubated in 1 and 10 µM of OA (Fig. 1A). There was no difference between OA treatments

Table 1. Effects of I-2 and OA on protein phosphatase activity in non-toxic (n=4) and toxic (n=3) mussel digestive gland tissue and rainbow trout tissue extracts (n=3). Phosphatase activity is expressed as pmol/min/mg protein ± SE, (percentage of control activity). * marks significantly lower activity from the control value ($p<0.05$). Assays were performed at 200-fold dilution in mussel tissue extracts and 2000-fold dilution in rainbow trout extracts, giving a final control activity of appr. 0.1 mU/ml.

Inhibitors	Non-toxic mussel	Toxic mussel	Rainbow trout
Control	339 ± 82 (100)	109 ± 20 (100)	1043 ± 208 (100)
200 nM I-2	165 ± 38 (48)	53 ± 6* (48)	388 ± 109* (37)
2 nM OA	169 ± 21* (50)	91 ± 6 (83)	647 ± 78 (62)
I-2 + OA	65 ± 19* (19)	38 ± 5* (35)	231 ± 10* (22)

and control value at 4 hours, and no trend towards a lower activity in 10 µM OA compared to 1 µM could be seen; GS activity was significantly increased in the 4 hour control treatment compared to the start value as seen previously in the absence of OA.

Rainbow trout slices were incubated in 10 µM OA (Fig. 1B). Glycogen synthase activity was significantly lower at 6 hours in the OA treatments compared to the control. The activity of the initial value at 0 hours and the 6 hour OA treatment did not differ.

In the mussel feeding experiment, OA content was measured after 0, 2, 4 and 8 days of exposure to toxic *P. lima* and non-toxic *P. micans*. Okadaic acid accumulated from 0 at the start to 1.7 µg OA/g digestive gland on day 8

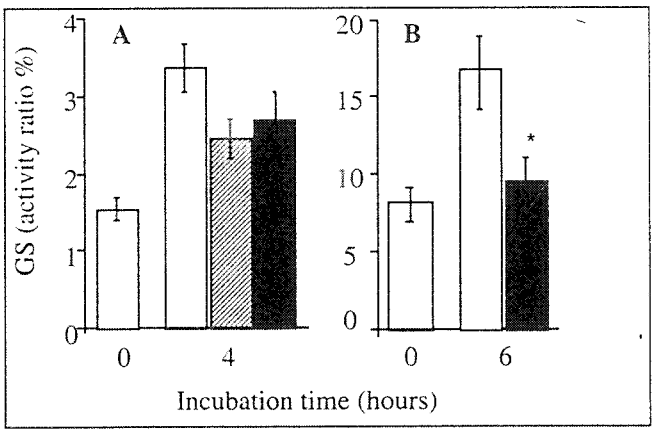

Fig. 1. Effects of OA on GS activity in (A): mussel digestive gland tissue (n=4) and (B): rainbow trout liver tissue (n=6). Unfilled bars: Controls. Striped bar: 1 µM OA. Filled bars: 10 µM OA.

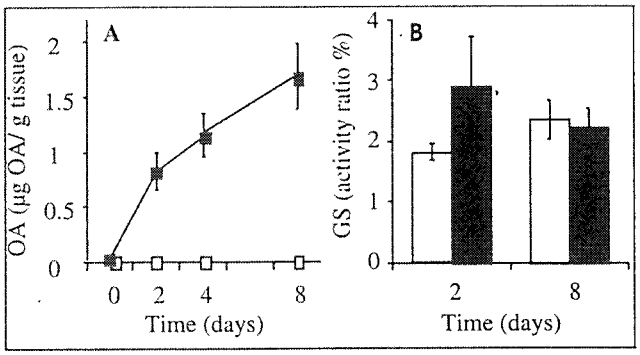

Fig. 2. (A): OA accumulation in mussels fed with *P. lima* (filled squares) and *P. micans* (unfilled squares). (B): GS activity in mussels fed with *P. micans* (unfilled bars) and *P. lima* (filled bars) (n=6).

in the mussels that had been fed the toxic algae (Fig. 2A). GS activity was measured after 2 and 8 days of feeding since OA content differed significantly between these sampling times. There was no difference in activity between mussels exposed to *P. lima* compared to *P. micans*, either in the 2 day or the 8 day treatment (Fig. 2B). Glycogen synthase activity levels in this feeding experiment were in the same range (2-3 %) as those in the *in vitro* study of mussel tissue.

DISCUSSION

We examined the intracellular effects of OA in the blue mussel, an organism frequently exposed to OA in nature, and rainbow trout, a species which generally never experiences this compound. In both species, PP activity was inhibited by 200 nM I-2 as well as 2 nM OA suggesting the presence of both PP1 and PP2A. Since PP activity measurements revealed equal sensitivity to I-2 and OA as other organisms that have been studied [8, 9, 21], these results provide further evidence for the observation of a conserved structure of these enzymes through eukaryotic evolution [11]. When phosphatase activity was measured in tissue from toxic mussels containing OA, the addition of 2 nM OA did not reduce the activity as in non-toxic mussel tissue. This seems to indicate that phosphatase activity is blocked by OA present in the tissue and thus could not be inhibited by further addition of 2 nM OA in the *in vitro* assay. These results show that OA may cause disturbances of the phosphorylation state of enzymes in these species.

We have used the activity of GS as a model enzyme to study the effects of OA on enzyme phosphorylation state. In blue mussels, the results from the *in vitro* tissue slice study showed that GS activity was not affected by 1-10 µM of OA. However, in rainbow trout tissue, there was a clear effect of 10 µM OA indicating the inhibition of convertion from the inactive state to the active state of GS by the action of OA on PP activity. This effect of OA in rainbow trout is consistent with results from mammalian cell culture studies where OA has been used to inhibit GS activation [5, 16].

The results from the mussel *in vitro* study were consistent with the results from the *in vivo* mussel feeding experiment. The mussels fed with the OA-producer *P. lima* accumulated OA (0.7 µg OA/g digestive gland on day 2 - 1.7 µg OA/g on day 8) corresponding to concentrations seen in Swedish mussel populations during blooms of toxic dinoflagellates. These amounts of OA corresponds to 0.83-

2.01 μM OA which should be high enough to fully inhibit the activity of PP1 and PP2A. However, these concentrations of OA did not alter GS activity compared to controls fed the same amount of the non-toxic *P. micans*. These shows that GS activity is not affected by OA at concentrations that naturally occurs in blue mussels *in vivo*.

On the basis of these results, it is suggested that there may be protective mechanisms against harmful effects of OA in the blue mussel. Of particular interest is that digestive cells of molluscan hepatopancreas are particularily rich in lysosomes [24]. It has been shown that lysosomes can accumulate and sequester foreign compounds such as lipophilic xenobiotics by increased autophagic activitiy [25]. Future studies will include tissue localization and lysosomal responses to OA which may help to further understand the survival strategies of blue mussels during blooms of DST-producers.

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REFERENCES

1. B. Underdal, M. Yndestad and T. Aune, in: Toxic Dinoflagellates, D.M. Anderson, A.W. White and D.G. Baden, eds. (Elsevier, New York), pp. 489-495 (1985).
2. E. Dahl and M. Yndestad, in: Toxic Dinoflagellates, D.M. Anderson, A.W. White and D.G. Baden, eds. (Elsevier, New York), pp. 495-500 (1985).
3. M. Kumagai, T. Yanagi, M. Murata, T. Yasumoto, M. Kat, P. Lassus and J.A. Rodríguez-Vázquez, Agric.Biol.Chem., 50, 2853-2857 (1986).
4. C. Bialojan and A. Takai, Biochem. J., 256, 283-290 (1988).
5. T.A.J. Haystead, A.T.R. Sim, D. Carling, R.C. Honnor, Y. Tsukitani, P. Cohen and D.G. Hardie, Nature, 337, 78-81 (1989).
6. M. Suganuma, H. Fujiki, H. Suguri, S. Yoshizawa, M. Hirota and M. Nakayasu, Proc. Nat. Ac. Sci., 85, 1768-1771 (1988).
7. K.N. Schmidt, E.B. Traenckner, B. Meier and P.A.J. Baeuerle, Biol. Chem., 270, 27136-27142 (1995).
8. P. Cohen, D.L. Schelling and M.J.R. Stark, FEBS letters, 250-2, 601-606 (1989).
9. C. MacKintosh and P. Cohen, Biochem. J. 262, 335-339 (1989).
10. M.P. Boland, M.F.J.R. Taylor and C.B.F. Holmes, FEBS Letter, 334, 13-17 (1993).
11. P. Cohen and P.T.W. Cohen, J. Biol. Chem., 264, 21435-21438 (1989).
12. P. Cohen and C.B.F. Holmes, Y. Tsukitani, Trends Biochem. Sci. 15, 98-102 (1990).
13. A.G. Bauder, A.D. Cembella and M.A. Quilliam. in: Harmful and Toxic Algal Blooms, T. Yasumoto, Y. Oshima and Y. Fukuro, eds. (Intergovernmental Oceanographic Commission of UNESCO) pp. 433-436 (1996).
14. S. Pillet, A. Pereira, J.C. Braekman and G. Houvenaghel, in: Harmful Marine Algal Blooms, P. Lassus, G. Arzul, E. Erard-Le DEn, P. Gentein and C. Marcaillou-Le Baut, eds. (Lavoisier Publishing, Paris) 487-492 (1995).
15. P.A. Cook and P.A. Gabbot, Comp. Biochem. Physiol., 60B, 419-421 (1978).
16. S. Pugazhenthi, B. Yu, R.R. Gali and R.L. Khandelwal, Bioch. et Biophys. Acta, 1179, 271-276 (1993).
17. M.A. Whittle and P.A. Gabbot, Comp. Biochem. Physiol., 83:B, 209-214 (1986).
18. J.S. Lee, T. Yanagi, R. Kenma and T. Yasumoto, Agr. Biol. Chem., 51, 877-881 (1987).
19. L. Edebo, S. Lange, X.P. Li, S. Allenmark, K. Lindgren and R. Thompson, APMIS, 96, 1036-1042 (1988).
20. P. Cohen, S. Alemany, B. Hemmings, T. Resink, P. Strålfors and H.Y. Limtung, Methods in Enzymology, 159, 390-409 (1988).
21. P. Cohen, S. Klumpp and D.L. Schelling, FEBS letters, 250-2, 596-600 (1989).
22. J.A. Thomas, K.K. Schlender and J. Larner, J. Anal. Biochem., 25, 486-499 (1968).
23. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J.J. Randall, Biol. Chem., 193, 265-275 (1951).
24. G. Owen, Sci. Prog. Oxf., 60, 299-318 (1972).
25. M.N. Moore, Mar. Ecol. Prog. Ser., 46, 81-89 (1988).

II

MUSSEL BLOOD CELLS, RESISTANT TO THE CYTOTOXIC EFFECTS OF OKADAIC ACID, DO NOT EXPRESS CELL MEMBRANE P-GLYCOPROTEIN ACTIVITY (MULTIXENOBIOTIC RESISTANCE)

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Abstract: Okadaic acid (OA) is a dinoflagellate toxin, accumulating in shellfish and causing Diarrhoeic Shellfish Poisoning (DSP) in humans. OA is a highly cytotoxic agent in most cell lines because of its inhibiting properties of protein phosphatases. So far, the cytotoxicity of OA in mussels, the main vectors of DSP, has not been investigated. In this paper, the viability of mussel (*Mytilus edulis*) blood cells incubated in 10 nM-1 µM OA was studied. After 72 hours of exposure, viability was reduced to 54% in 1 µM OA compared to 88% in control cells. This yielded a LC_{50} of >1 µM for OA, which is 30-1000 times higher compared to other cell types. We tested if P-glycoprotein (p-gp) activity (multixenobiotic resistance, MXR) contributed to the resistance to OA. Vincristine and rhodamine B was used as p-gp substrates and verapamil or staurosporine as inhibitors of p-gp transport. However, we found no indications of cell membrane p-gp activity. Instead, experimental observations led to the conclusion that a MXR transport system was present within lysosomal membranes. Various concentrations of OA did not affect the dynamics of vincristine in blood cells. As a positive control for the assay, p-gp activity was measured in mussel gill tissue. The efflux of rhodamine B was reduced by verapamil which is considered evidence for cell membrane p-gp activity, thus the accuracy of the method was confirmed. Rhodamine B efflux was also reduced by OA in gill tissue, which suggested that OA is either a competitive substrate or inhibitor of p-gp activity. When the volume of the lysosomal compartment was measured in blood cells pre-exposed to OA, a significant increase was detected compared to control cells. We propose that uptake and storage of OA within the lysosomal system might protect mussel blood cells from the cytotoxic effects of this compound.

Keywords: Mytilus edulis, blood cells, okadaic acid, MXR, p-glycoprotein, lysosomes.

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1. Introduction

Okadaic acid (OA), the main causative agent of diarrhoeic shellfish poisoning (DSP) in humans, is a polyether fatty acid produced by marine dinoflagellates (Tachibana et al., 1981; Murata et al., 1982; Yasumoto et al., 1984). This algal toxin is a specific inhibitor of serine/threonine phosphatases, predominantly protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Bialojan and Takai 1988). By blocking the activity of PP1 and PP2A, OA inhibits dephosphorylation of numerous enzymes which in turn causes major effects on signal transduction pathways and hence essential cellular functions (Haystead et al., 1989; Fujiki and Suganuma, 1993). Low concentrations of OA (nanomolar) have been found to be cytotoxic in a vast range of cell culture systems and induce apoptotic cell death (Boe et al., 1991; Kawamura et al., 1996; Ritz et al., 1997; Fladmark et al., 1998).

Although intensively studied in various mammalian systems, the cytotoxicity and impact of OA in molluscs, the main vectors for DSP, has been given little attention. Some mussels, such as the blue mussel, *Mytilus edulis*, along the Swedish west coast, are exposed to OA during prolonged periods each year (Edebo et al., 1988; Lindegarth, 1997). Even so, no apparent increase in mortality within populations of mussels has been observed. Thus, it seems likely that organisms, which are naturally exposed to toxins such as OA, have some protective mechanisms against its harmful effects. Svensson and Förlin (1998) identified both PP1 and PP2A in the digestive gland of blue mussels to be just as sensitive to *in vitro* inhibition by OA as other studied organisms. Although highly sensitive to OA on a molecular level, no *in vivo* effects of OA on a cytosolic enzyme (glycogen synthase), regulated by PP1 and PP2A, were detected. It was suggested that mussels might have mechanisms which prevent OA from accumulating within the cytosolic environment.

The apparent lack of intracellular effects of OA could be due to multixenobiotic resistance (MXR) in mussel cells. MXR is a defence mechanism in non-mammalian organisms which is similar to multidrug resistance (MDR), a term used in cancer research to describe tumour cells resistant to cytotoxic drugs (MXR reviewed by Bard, 2000). This resistance is commonly due to overexpression of a plasma membrane phosphoglycoprotein (p-gp) which function as an ATP-dependent drug efflux pump, reducing intracellular levels of various, structurally unrelated, lipophilic compounds (MDR review, see Endicott and Ling 1989; Ambudkar et al., 1999). Some MDR cell lines have been found to be resistant to the cytotoxic effects of OA (Chambers et al., 1993). Also, cell clones selected for resistance to OA show a MDR phenotype (Tohda et al., 1994; Wang et al., 1995; Ritz et al., 1997). One mechanism involved in the resistance is probably due to an increased extrusion of OA, since resistance can be reversed by the addition of verapamil, a competitive inhibitor of p-gp activity (Yusa and Tsuruo 1989; Tohda et al., 1994; Ritz et al. 1997). In *Mytilus* sp., p-gp expression and activity has been found in gill and mantle tissue (Minier et al., 1993; Cornwall et al., 1995; Kurelec, 1995), in isolated membrane vesicles of the digestive gland (Kurelec and Pivcevic', 1991) and in blood cells (Minier and Moore 1996).

An alternative protective mechanism would be the uptake and storage of OA in the lysosomal system (Svensson and Förlin 1998). Many of the molluscan cell types are particularly rich in lysosomes (Moore, 1990) and these organelles are known to concentrate a wide range of foreign compounds, such as lipophilic xenobiotics and heavy metals. Thereby the rest of the cell is protected from any deleterious effects of these substances. Thus, a highly developed lysosomal system represents a mechanism by which cells can be

more tolerant to some pollutant stress (Moore and Willows 1998).

In this paper, the cytotoxicity of OA in mussel blood cells was studied. We found that these cells were highly resistant against the cytotoxic effects of OA. Therefore, we tested if MXR might play a role in this resistance. We also performed some preliminary studies on the effects of OA on the lysosomal compartment.

2. Materials and Methods

2.1. Animals

Adult *M. edulis* (7-10 cm shell height, mean dry weight 6 g) were collected in 1998 from Inre Vattenholmen, off Tjärnö Marine Biological Laboratory on the northern part of the Swedish west coast, during a period of non-detectable levels of OA in the mussels. They were returned to the laboratory and maintained in flow-through tanks with the addition of *Isochrysis galbani*.

2.2. Extraction of mussel blood

Hemolymph was withdrawn from the posterior adductor muscle into a sterile 5 ml hypodermic syringe fitted with a 25 mm needle. The blood was immediately diluted with an equal volume of physiological saline (PS) (20 mM HEPES, 436 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 53 mM $MgSO_4$, pH 7.3) and put on ice. Cell concentration was determined by staining cells 1:5 with Gentiana violet (40 µl blood to 160 µl staining solution) and counted in a light microscope at x40 magnification using a hemocytometer. Mean cell concentration for the sampled mussel population was $2.1 \pm 0.8*10^6$ cells ml^{-1}. In all experiments, the following protocol was used: hemolymph suspensions from three individuals were pooled, cell concentration was determined and the suspension was further diluted in PS to a final concentration of $2*10^5$ cells ml^{-1}. 10^5 cells (0.5 ml) were seeded into wells of a sterile 24-well tissue culture plate (Falcon). The cells were left to attach to the bottom surface for 30 min and the overlying solution was removed by a Pasteur pipette. 0.5 ml of fresh PS was added with or without various drugs as described below.

2.3. Cytotoxicity assay

OA (1 mM stock solution in DMSO, Sigma) was diluted in PS supplemented with 2% (w/v) glucose and 1% (v/v) antibiotics (gentamicin, 10 mg ml^{-1}, Sigma). The blood cells were exposed to various concentrations of OA (10 nM to 1 µM) in 0.5 ml of supplemented PS for 24 to 72 hours. As controls, cells were incubated in the OA vehicle (DMSO). The microplates were incubated in 10° C in a light-proof humidity chamber. After each incubation period, the cells were detached from the surface by adding 0.25% (w/v) trypsin (bovine pancreas, 12.400 Units mg^{-1} protein, Sigma) and transferred to Eppendorf tubes. Cell viability vas determined using the red dye Eosin Y (Sigma). 20 µl of the cell suspension was mixed with an equal volume of 0.05% Eosin Y (w/v, dissolved in PS) and transferred to a hemocytometer. Viable (unstained) and non-viable (stained) cells were counted immediately and the viability was calculated as % viable cells of total cell count (minimum 200 cells counted).

2.4. Assay for p-gp activity in blood cells

The activity of p-gp was estimated by measuring the intracellular accumulation of the *Vinca* alkaloid vincristine (VCR), a substrate for the p-gp transporter. Radiolabelled G-^{3}H-vincristine sulphate (0.75 µCi/ml, Amersham) and unlabelled vincristine sulphate (Sigma) was mixed to a final concentration of 0.5 µM VCR in PS. As inhibitors of p-gp activity, 10-20 µM verapamil (VP) and 0.5 µM staurosporine

(ST) were used. The cells were incubated in 0.5 µM VCR for various time periods in 15° C in the dark in the absence (controls) or presence of the inhibitors. Also, the effects of OA (10 nM to 1 µM) on the accumulation of VCR was tested. At the end of each incubation period, the radioactive solution was removed and the cells were washed in 0.5 ml PS for one minute, which was repeated three times. The cells were lyzed by adding 0.5 ml of a solution containing 1 M NaOH and 5% (v/v) Triton 100-X for 2 hours. The solution was transferred to vials containing 4.5 ml scintillation liquid (Opti-phase 'HiSafe' 3, Wallac OY) and the radioactivity was counted. Also, the fluorescent substance rhodamine B (Rh B) was used as a p-gp substrate. Cells were incubated for 30 minutes in 4 µM rh B in the absence or presence of 10 and 20 µM VP. After the incubation period, cells were washed with PS three times as above, the intracellular Rh B was extracted by adding extraction solution (0.5 ml of 1% (v/v) acetic acid in 50 % ethanol for 10 minutes) and the fluorescence intensity was measured on a spectrofluorometer (Shimadzu RF-510) at excitation wavelength 546 nm and recording emission of 578 nm. Cells exposed to Rh B with or without VP were also studied in an epifluorescence microscope in order to qualitatively check the fluorescence intensity within the cells.

2.5. Assay for p-gp activity in gill tissue

Mussel gill tissue was used as a positive control for p-gp activity since the presence of a p-gp mediated transport system in this tissue has been widely documented (Minier et al. 1993; Cornwall et al. 1995, Kurelec, 1995, Eufemia and Epel, 2000). The gills from 15 individuals were cut out and put in ice cold PS. Mucus was removed and small sections of app. 25 mm^2 were cut by scissors and placed in a beaker of PS. 0.1 g (ww) of randomly chosen tissue pieces were placed in 2 ml PS and then Rh B was added to obtain 2 µM Rh B. The gill tissue was incubated for 30 minutes at 20°C in the absence (control) or presence of 20 µM VP or 10-100 nM OA. The tissue pieces were then transferred to new vials and washed three times in PS and after that placed in 2 ml of extraction solution for 15 minutes as described above for blood cells. The tissue was removed and discarded and the fluorescence intensity of the remaining solution was measured.

2.6. Assay for lysosomal volume

The lysosomal volume of cells incubated in various concentrations of OA vas measured by the Neutral Red (NR) uptake assay according to Grundy et al. (1996) with some modifications. The cells were incubated for 24 hours in 10 nM-1 µM OA in the same manner as in the cytotoxicity test described above. They were then washed in PS, 0.5 ml NR solution (50 µg NR ml^{-1} dissolved in PS) was added and the plates were incubated for 3 h in the dark. The cells were washed three times with PS and intracellular NR was extracted by adding the extraction solution as described for Rh B. Colour intensity was measured in a spectrophotometer at 540 nm.

2.6. Statistical tests

All data was analysed using one factor or two factor analysis of variance (ANOVA). Data was first checked for homogeneity of variances using Cochran´s test. For significant effects, Student-Newman-Keuls (SNK) aposteriori test for differences among means was used.

3. Results

3.1. Cytotoxicity of OA

The viability of blood cells incubated for 24, 48 and 72 hours in different concentrations of OA are shown in figure 1. Cells incubated in 1 µM OA showed a significant reduction in

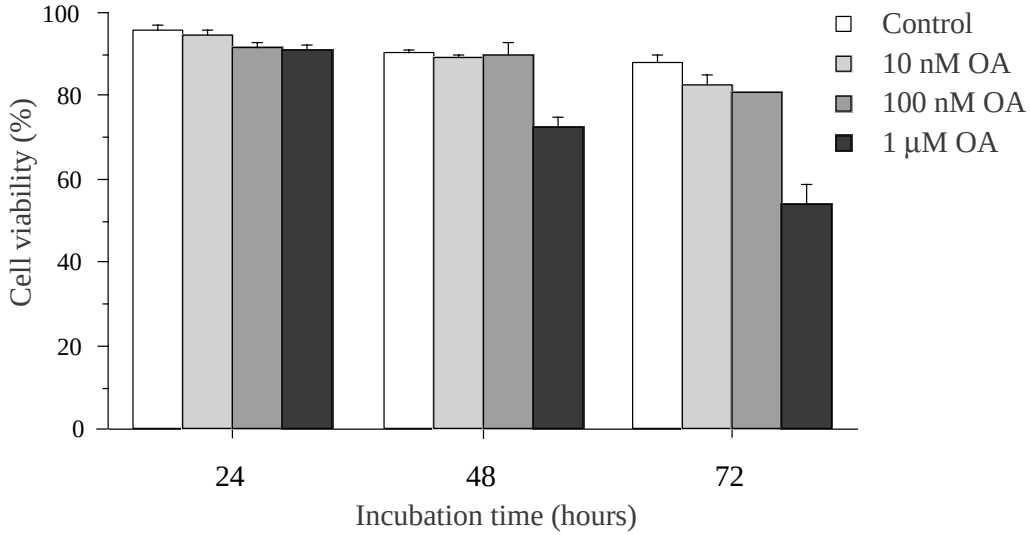

Fig. 1. Viability of mussel blood cells incubated in various concentrations of okadaic acid (OA) for 24 to 72 hours. As a control, cells were incubated in the OA solvent (DMSO). Cell viability was calculated as % viable cells of total cell count. Values are means ± SE (n=2).

viability in time compared to the other treatments (treatment*time, P <0.001). After 24 h, there was no difference in viability among treatments. However, viability was significantly reduced to 72% after 48 hours in 1 µM OA compared to controls (90%) and a further reduction to 54% was observed after 72 hours (control cells, 88% viability). Over time, there was no significant difference in viability for cells incubated in 10 and 100 nM

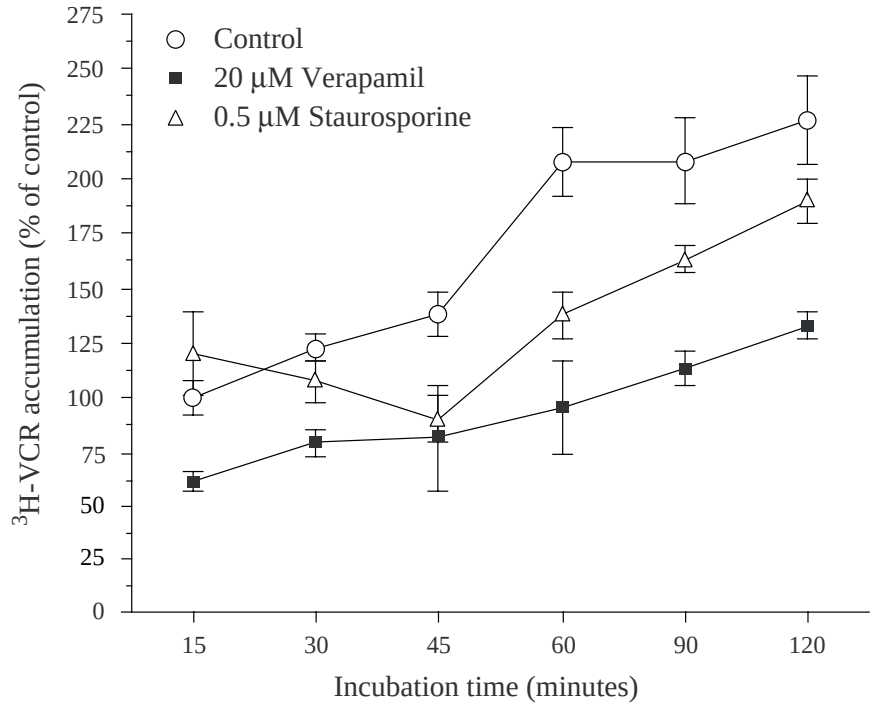

Fig. 2. Accumulation of ^{3}H-vincristine (^{3}H-VCR) in blood cells incubated for 15 to 120 minutes in the absence (control) and presence of 20 µM verapamil or 0.5 µM staurosporine. The mean intracellular ^{3}H-VCR content in control cells after 15 minutes of incubation was set at 100%. Values are means ± SE (n=2).

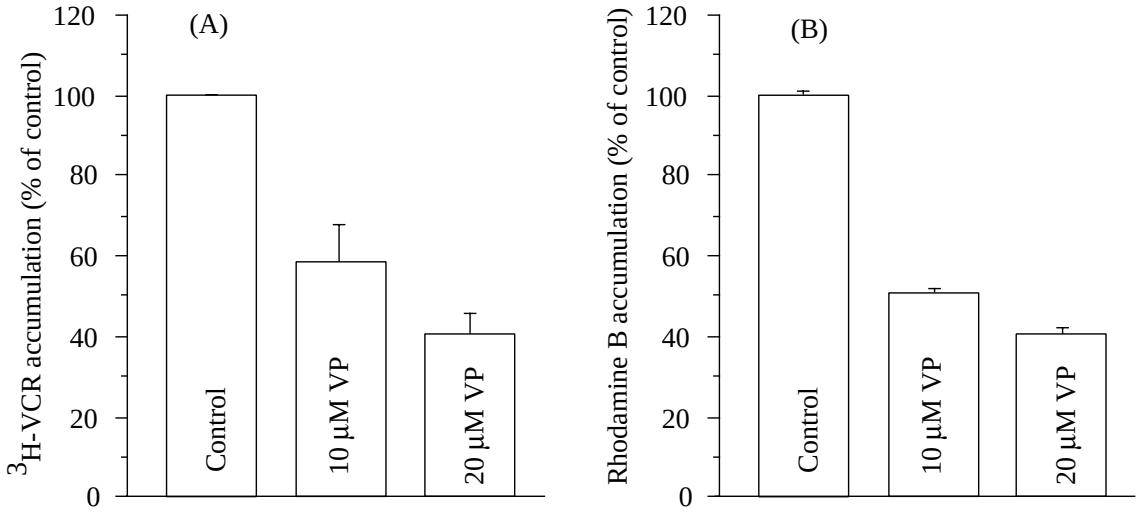

Fig. 3. Accumulation of ^{3}H-vincristine (^{3}H-VCR) (A) and rhodamine B (B) in blood cells incubated for 30 minutes in the absence (control) and presence of 10 and 20 µM verapamil. For each substrate, the mean intracellular content in the controls was set at 100%. Values are means ± SE (n=2).

compared to the control cells. Since the viability for blood cells incubated in 1 µM OA were >50% after 72 h, the LC_{50} value (concentration that reduces the number of viable cells to 50%) for OA was >1 µM in this system.

3.2. Test of p-gp activity in blood cells

Blood cells were exposed to 0.5 µM VCR in the absence and presence of 20 µM VP or 0.5 µM ST. The intracellular accumulation of VCR was measured in intervals of 15 minutes up to 120 minutes of drug exposure (fig. 2). The mean intracellular ^{3}H-VCR content in control cells after 15 minutes of incubation was set at 100%. There was a highly significant difference among treatments ($P < 0.001$) and among times ($P < 0.001$). In all treatments, accumulation of VCR increased in time. Cells treated with VP had a lower content of VCR at all times compared to the control (only VCR added) indicating that the effect of VP on accumulation of VCR was faster than 15 minutes of exposure. In the ST treatments, the content of VCR was lower compared to controls at 45 minutes and thereafter. Since this result was contradictory to the expected ”classical” MXR response to p-gp inhibitors, we performed additional tests of p-gp activity using Rh B as a substrate. Cells were exposed to 4 µM Rh B for 30 minutes in the absence or presence of 10-20 µM VP and compared to the accumulation of 0.5 µM VCR (fig. 3). The same pattern was again observed. For both substrates, the accumulation was significantly reduced in a dose-dependent manner in cells exposed to VP compared to the control ($P < 0.01$). The content of VCR in cells exposed to 10 µM VP was reduced to 59% compared to controls (100%) and further reduced to 40% in 20 µM VP. For Rh B, these values were 52 % and 40 % respectively. When cells exposed to Rh B were studied in the microscope, numerous fluorescent vesicles containing Rh B were seen inside the cells (data not shown). It was clear from the images that cells exposed to Rh B alone showed a higher fluorescence intensity compared to VP-treated cells which confirmed the results from the previous study as well as identifying these vesicles as the site of Rh B accumulation.

Blood cells were incubated in 0.5 µM VCR for 45 minutes in the absence (control) and presence of various combinations of OA (10, 100 nM and 1 µM), VP (20 µM) and ST (0.5 µM), (fig. 4). The accumulation of VCR was not affected by the addition of OA alone (fig. 4, control bars, $P > 0.05$). Also, OA in

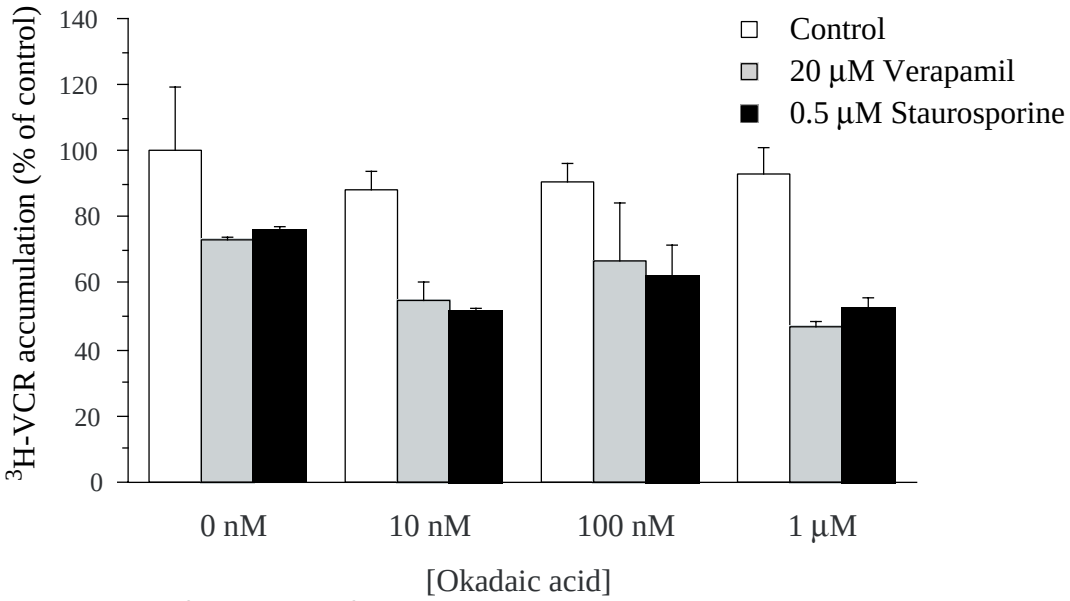

Fig. 4. Accumulation of ^{3}H-vincristine (^{3}H-VCR) in blood cells incubated for 45 minutes in the absence (control) and presence of various combinations of OA (10, 100 nM and 1 µM), verapamil (20 µM) and staurosporine (0.5 µM). The mean intracellular ^{3}H-VCR content in control cells was set at 100%. Values are means ± SE (n=2).

combination with VP or ST did not cause any significant change in the accumulation of VCR compared to VP or ST alone. The accumulation was significantly reduced by both inhibitors ($P < 0.001$) in the same manner as seen in previous experiments regardless of the presence of OA.

3.3. P-gp activity in gill tissue

Gill tissue was incubated in 2 µM Rh B for 30 minutes in the absence (control) or presence of 20 µM VP, 10 nM OA or 100 nM OA (Fig. 5). Compared to controls (100%), Rh B accumulation significantly increased in tissue incubated in 20 µM VP (132%) as well as in 10 nM OA (130%) and 100 nM OA (137%) ($P < 0.05$). This result indicated that gill tissue expressed cell membrane p-gp activity since the efflux of Rh B was inhibited by VP. Also, since Rh B efflux was similarly reduced by OA, this suggested that OA interact with the p-gp transporter either by being a competitive substrate or an inhibitor of p-gp activity.

3.3. Lysosomal volume

Cells were exposed to 10, 100 nM and 1 µM OA for 24 hours whereafter the volume of the lysosomal compartment was estimated as the uptake of Neutral Red (Table 1). Compared to control cells, the lysosomal volume increased significantly in 10 nM OA (25%) and further in 100 nM (42%) ($P < 0.05$, Table 1). In cells treated with 1 µM OA, a slight but not significant increase in the lysosomal volume compared to control cells was found (10%).

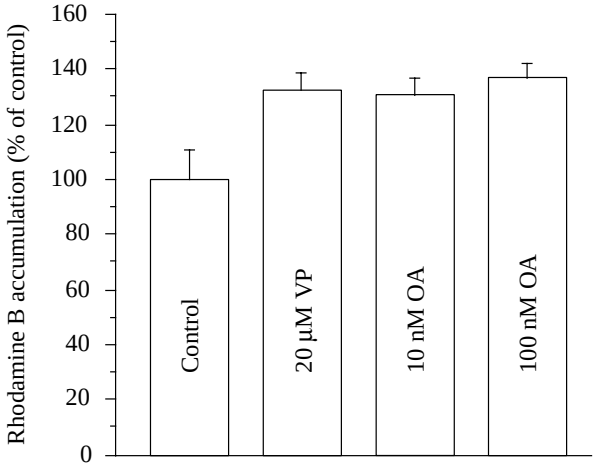

Fig. 5. Accumulation of rhodamine B (2µM) in mussel gill tissue in the absence (control) or presence of 20 µM verapamil (VP), 10 nM okadaic acid (OA) and 100 nM OA. The mean fluorescence intensity in control tissue was set at 100 %. Values are means ± SE (n=5).

4. Discussion

The cytotoxicity of OA, a potent serine/threonine phosphatase inhibitor, has been studied in various cell culture systems where it is generally regarded as a highly toxic substance. Prolonged inhibition of PP1 and PP2A triggers the signalling cascade for apoptotic cell death, which seems to be the underlying mechanism for the cytotoxicity of OA (Ritz et al. 1997). LC_{50} values reported for OA range from 1.5 nM in human epidermoid carcinoma cells (KB) cells (Tubaro et al., 1996), 13 nM in Chinese hamster ovary cells (CHO-K1) (Tohda et al. 1994), 20 nM in salmon hepatocytes (Fladmark et al., 1998), 30 nM in rat pituitary GH_3 cells (Ritz et al. 1997) and 31 nM in mouse neuroblastoma cells (N1E-115) (Laidley et al., 1997). In this study, mussel blood cells exposed to 1 µM OA showed a reduction in viability compared to our control treatments. However, >50% of the cells were still viable after 3 days of exposure to 1 µM OA, yielding a LC_{50} >1 µM in this system. Considering the low LC_{50} values previously reported for OA, mussel blood cells seemed to be highly resistant to the cytotoxic effects of OA, surviving 30 to 1000 times greater concentrations compared to other cell types.

We proposed that MXR in the form of p-gp activity in the cell membranes might be involved in the protection against the toxic effects of OA in mussel blood cells, reducing the intracellular levels of OA. This assumption stemmed from observations in the literature where OA evidently is a substrate for p-gp in various MDR cell lines (Tohda et al. 1994; Ritz et al. 1997). Using VCR, an extensively documented substrate of p-gp and the p-gp inhibitors VP and ST, we initially aimed to characterise cell membrane p-gp activity in blood cells. Both VP, a calcium channel blocker, and ST, an inhibitor of protein kinase C, have been shown to interact directly with the p-gp molecule, thereby inhibiting the efflux of VCR (Budworth et al., 1996; Ford 1996). We then aimed to measure the uptake of VCR in the presence of OA and predicted a reduced efflux of VCR due to competition of the p-gp binding site. Thus, our findings that the accumulation of VCR was reduced in the presence of both inhibitors was contrary to the predicted effects of VP and S. Additionally, when we used Rh B as a p-gp substrate, the accumulation was similarly reduced in a dose-dependent manner by VP. When blood cells were exposed to various concentrations of OA, this did not significantly affect the dynamics of VCR.

Table 1. Uptake of Neutral Red (NR) as a measure of lysosomal volume in blood cells pre-exposed to various concentrations of OA for 24 hours. As a control, cells were incubated in the OA solvent (DMSO). Accumulation of NR in the control treatment was set at 100%. Values are means ± SE (n=3).* indicates P <0.05 compared to control.

[Okadaic acid]	Uptake of NR (% of control)
Control	100 ± 0.4
10 nM	125 ± 7.8*
100 nM	142 ± 1.1*
1 µM	110 ± 7.7

Our results strongly indicated that mussel blood cells used in this study did not express 'classical' p-gp activity in the plasma membranes. These results are in contrast to those found by Minier and Moore (1996) in *M. edulis* blood cells. They confirmed the presence of a 170 kDa MDR-like protein in blood cells using the monoclonal antibody C219. When we repeated their assay system for p-gp activity using the same concentrations of both Rh B (4 µM) and VP (20 µM), we obtained a reduced accumulation of Rh B in the presence of VP which was opposite to their findings. This result was puzzling. We therefore performed additional experiments using mussel gill tissue to serve as a positive control for our p-gp assay system and also to test if OA may be a substrate for

the p-gp transporter. We found that Rh B efflux in this tissue was in fact inhibited by VP, which is considered evidence of cell membrane p-gp activity (Bard 2000). The addition of OA similarly reduced the efflux of Rh B which suggested that this toxin might actually be a competitive substrate for p-gp or alternatively an inhibitor of p-gp activity in mussel gill tissue. However, this kind of p-gp assay system only yields indirect evidence for OA transport by p-gp and more evidence is needed to conclude that OA is in fact a p-gp substrate in mussel gill tissue. Ideally, studies of p-gp activity using direct measurements of intracellular OA levels should be performed. Although Tohda et al. (1994) and Ritz et al. (1997) provided convincing evidence that OA is a p-gp substrate in MDR cell lines, it should also be noted that this is perhaps not always the case. Wielinga et al., (1997) treated MDR and wildtype cells with 200 nM OA which did not change the accumulation of three different substrates of p-gp, suggesting that OA does not affect p-gp activity.

A possible explanation to our observations in blood cells is that the main site for p-gp activity was within subcellular membranes. Intracellular MXR transport is suggested by Moore and Willows (1998) as a mode of accumulation of toxic pollutants from the cytosol into subcellular organelles. In the crab *Carcinus maenas*, p-gp expression has been found in lysosomal membranes of F/B cells in the digestive gland (Köhler et al., 1998). Microscopical examination of cells exposed to Rh B in this study confirmed that this compound accumulated within intracellular organelles and this accumulation was inhibited by VP which could be seen as a reduced fluorescence intensity within the organelles. Minier and Moore (1996) identified organelles containing Rh B within mussel blood cells to be lysosomes since they co-stained with the lysosomal-specific dye Neutral Red. Altogether, our results indicated that the lysosomal membranes were the main sites for a p-gp-related MXR transport system in this study.

To explain our results compared to those obtained by Minier and Moore (1996), it may be possible that the two mussel populations, from the English and Swedish coast respectively, have evolved unique p-gp expression patterns. Variability in several environmental factors, for example contaminants, salinity and tidal height, among those habitats could possibly induce different patterns of p-gp expression and activity which would be of interest to investigate further.

A comparison to human blood cells show that p-gp expression is not always connected to the plasma membrane. Klimecki et al., (1994) found that granulocytes (CD15+) expressed p-gp in membrane fractions but despite this, the granulocytes lacked both measurable efflux transport of a p-gp substrate as well as cell-surface staining by two different monoclonal anti-p-gp. This pattern was also found by Drach et al. (1992). *Mytilus* sp. blood cells (granulocytes and hyalinocytes) and human granulocytes show several immunological similarities such as conformational changes and locomotory responses following exposure to opioid peptides (Stefano et al., 1989). Considering these similarities between mussel blood cells and human granulocytes, it seems possible that these cell types also may show similar patterns of p-gp expression and activity.

Whether or not OA is a substrate for the proposed lysosomal p-gp-related activity in mussel blood cells remains unclear from our study. Nevertheless, when the volume of the lysosomal compartment was measured in cells pre-exposed to OA, a significant increase was detected compared to our control cells where the largest increase were found in cells incubated in 100 nM OA. This suggests that OA affect the lysosomal system, possibly by accumulating within the lysosomes which causes swelling of these organelles. Exposing cells to 1 µM OA may have reduced the lysosomal membrane integrity causing

leakage of Neutral Red and possibly other components within the lysosomes, including OA. The lysosomal system is the main target for toxicants in molluscs where the most documented deleterious effect concerns the destabilisation of the membrane structure (Lowe et al., 1995; Grundy et al. 1996; Ringwood et al., 1998). Thus, structural changes in the lysosomal membranes with subsequent failure to retain OA within the lysosomes could explain why the viability was reduced in cells exposed to 1 µM OA but not 10 or 100 nM.

In conclusion, we found no support for the model that mussel blood cells express p-gp activity in the cell membranes which would contribute to the high resistance to the cytotoxic effects of OA. Instead, we propose that the lysosomal system might function as a protective mechanism against OA in the blood cells. Future experiments will include lysosomal response to OA in both digestive gland cell cultures as well as further studies in blood cells. Analysis of OA content in subcellular fractions of mussel tissue will also be performed in order to determine intracellular localisation of OA.

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References

Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman, M.M., 1999. Biochemical, cellular and pharmacological aspects of the multidrug transporter. Ann. Rev. Pharmacol. Toxicol. 39, 361-398.

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defence mechanism in aquatic organisms. Aquat. Toxicol. 48, 357-389.

Bialojan, C., Takai, A., 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Biochem. J. 256, 283-290.

Boe, R., Gjertsen, B.T., Vintermyr, O.K., Houge, G., Lanotte, M., Doskeland, S.O., 1991. The protein phosphatase inhibitor okadaic acid induces morfological changes typical of apoptosis in mammalian cells. Exp. Cell Res. 195, 237-246.

Budworth, J., Davies, R., Malkhandi, J., Gant, T.W., Ferry, D.R., Gescher, A., 1996. Comparison of staurosporine and four analogues: their effects on growth, rhodamine 123 retention and binding to P-glykoprotein in multidrug-resistant MCF-7/Adr cells. Brit. J. Canc. 73, 1063-1068.

Chambers, T.C., Raynor, R.L., Kuo, J.F., 1993. Multidrug-resistant human KB carcinoma cells are highly resistant to the protein phosphatase inhibitors okadaic acid and calyculin A. Analysis of potential mechanisms involved in toxin resistance. Int. J. Canc. 53, 323-327.

Cornwall, R., Holland Toomey, B., Bard, S., Bacon, C., Jarman, W.M. & Epel, D., 1995. Characterization of multixenobiotic/multidrug transport in the gills of the mussel *Mytilus californianus* and identification of environmental substrates. Aquat. Toxicol. 31, 277-296.

Drach, D., Zhao, S., Drach, J., Mahadevia, R., Gattringer, C., Huber, H., Andreeff, M., 1992. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. Blood. 80, 2729-2734.

Edebo, L., Lange, S., Li, X.P., Allenmark, S., Lindgren, K., Thompson, R., 1988. Seasonal, geographic and individual variation of okadaic acid content in cultivated mussels in Sweden. APMIS. 96, 1036-1042.

Endicott, J.A., Ling, V., 1989. The biochemistry of P-glycoprotein-mediated

multidrug resistance. Ann. Rev. Biochem. 58, 137-171.

Eufemia, N.A., Epel, D., 2000. Induction of the multixenobiotic defence mechanism (MXR), P-glycoprotein, in the mussel *Mytilus californianus* as a general cellular response to environmental stress. Aquat. Toxicol., 49, 89-100.

Fladmark, K.E., Serres, M.H., Larsen, N.L., Yasumoto, T., Aune, T., Döskeland, S.O., 1998. Sensitive detection of apoptogenic toxins in suspension cultures of rat and salmon hepatocytes. Toxicon. 36(8), 1101-1114.

Ford, J.M., 1996. Experimental reversal of P-glukoprotein-mediated multidrug resistance by pharmacological chemosensitisers. Eur. J. Canc. 32A, 991-1001.

Fujiki, H., Suganuma, M., 1993. Tumor promotion by inhibitors of protein phosphatase 1 and 2A: the okadaic acid class of compounds. Adv. Canc. Res. 61, 143-194.

Grundy, M.M., Moore, M.N., Howell, S.M., Ratcliffe, N.A., 1996. Phagocytic reduction and effects on lysosomal membranes of polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. Aquat. Toxicol. 34, 273-290.

Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P., Hardie, D.G., 1989. Effects of the tumour-promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature. 337, 78-81.

Kawamura, K.-I., Grabowski, D., Weizer, K., Bukowski, R., Ganapathi, R., 1996. Modulation of vinblastine cytotoxicity by dilantin (phenytoin) or the protein phosphatase inhibitor okadaic acid involves the potentiation of anti-mitotic effects and induction of apoptosis in human tumor cells. Brit. J. Cancer. 73, 183-188.

Klimecki, W.T., Futscher, B.W., Grogan, T.M., Dalton, W.S., 1994. P-glycoprotein expression and function in circulating blood cells from normal volunteers. Blood. 83, 2451-2458.

Kurelec, B., 1995. Reversion of the multixenobiotic resistance mechanism in gills of a marine mussel *Mytilus galloprovincialis* by a model inhibitor and environmental modulators of P170-glycoprotein. Aquat. Toxicol. 33, 93-103.

Kurelec, B., Pivcevic', B., 1991. Evidence for a multixenobiotic resistance mechanism in the mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 19, 291-302.

Köhler, A., Lauritzen, B., Jansen, D., Böttcher, P., Teguliwa, L., Krüner, G., Broeg, K., 1998. Detection of P-glycoprotein mediated MDR/MXR in *Carcinus maenas* hepatopancreas by immuno-gold-silver labeling. Mar. Env. Res. 48, 411-414.

Laidley, C.W., Cohen, E., Casida, J.E., 1997. Protein phosphatase in neuroblastoma cells: ^{3}H-cantharidin binding site in relation to cytotoxicity. J. Pharmacol. Exp. Ther. 280, 1152-1158.

Lindegarth, M., 1997. Sammanställning och tolkning av de kontinuerliga mätningarna av okadasyrahalter längs den svenska västkusten. Bohuskustens vattenvårdsförbund, Göteborg (In swedish).

Lowe, D.M., Soverchia, C., Moore, M.N., 1995. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquat. Toxicol. 33, 105-112.

Minier, C., Akcha, F., Galgani, F., 1993. P-glycoprotein expression in *Crassostrea gigas* and *Mytilus edulis* in polluted seawater. Comp. Biochem. Physiol. 106B, 1029-1036.

Minier, C., Moore, M.N., 1996. Rhodamine B accumulation and MXR protein expression in mussel blood cells: effects of exposure to vincristine. Mar. Ecol. Prog. Ser. 142, 165-173.

Moore, M.N., 1990. Lysosomal cytochemistry in marine environmental monitoring. Histochem. J. 22, 187-191.

Moore, M.N., Willows, R.I., 1998. A model for cellular uptake and intracellular behaviour of particulate-bound micropollutants. Mar. Env. Res. 46, 509-514.

Murata, M., Shimatani, M., Sugitani, H., Oshima, Y., Yasumoto, T., 1982. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. Bull. Jap. Soc. Sci. Fish. 48, 549-552.

Ringwood, A.H., Conners, D.E., Houget, J., 1998. Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters *Crassostrea virginica*. Mar. Ecol. Prog. Ser. 166, 163-171.

Ritz, V., Marwitz, J., Richter, E., Ziemann, C., Quentin, I., Steinfelder, H.J., 1997. Characterization of two pituitary GH3 cell sublines partially resistant to apoptosis induction by okadaic acid. Biochem. Pharmacol. 54, 967-971.

Stefano, G.B., Leung, M.K., Zhao, X., Scharrer, B., 1989. Evidence for the involvement of opioid neuropeptides in the adherence and migration of immunocompetent invertebrate blood cells. Proc. Nat. Acad. Sci. USA. 86, 626-630.

Svensson, S., Förlin, L., 1998. Effects of okadaic acid on protein phosphatase and glycogen synthase activities in the blue mussel, *Mytilus edulis*, and rainbow trout, *Oncorhynchus mykiss*. In: Reguera, B., Blanco, J., Fernandez, M. L., Wyatt, T. (Eds.), Harmful algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 584-587.

Tachibana, K., Scheuer, P.J., Tsukitani, Y., Kikuchi, H., Van Eugen, D., Clardy, J., Gopichand, Y., Schmitz, F.J., 1981. Okadaic acid, a cytotoxic polyether toxin from two marine sponges of the genus *Halichondria*. J. Am. Chem. Soc. 103, 2469-2471.

Tohda, H., Yasui, A., Yasumoto, T., Nakayasu, M., Shima, H., Nagao, M., Sugimura, T., 1994. Chinese Hamster ovary cells resistant to okadaic acid express a multidrug resistant phenotype. Biochem. Biophys. Res. Com. 203, 1210-1216.

Tubaro, A., Florio, C., Luxich, E., Vertua, R., Della Loggia, R., Yasumoto, T., 1996. Suitability of the MTT-based cytotoxicity assay to detect okadaic acid contamination of mussels. Toxicon. 34, 965-974.

Wang, S.J., Scavetta, R., Lenz, H.J., Danenberg, K., Danenberg, P.V., Schonthal, A.H., 1995. Gene amplification and multidrug resistance induced by the phosphatase-inhibitory tumor promoter, okadaic acid. Carcinogenesis 16, 637-641.

Wielinga, P.R., Heijn, M., Broxterman, H.J., Lankelma, J., 1997. P-glycoprotein-independent decrease in drug accumulation by phorbol ester treatment of tumor cells. Biochem. Pharmacol. 54, 791-799.

Yasumoto, T., Murata, M., Oshima, Y., Matsumoto, G.K., Clardy, J., 1984. Diarrhetic shellfish poisoning. In: Ragelis, E. P. (Ed.), Seafood toxins. ACS Symp. Ser. 262, Washington D.C., pp. 207-214.

Yusa, K., Tsuruo, T., 1989. Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. Canc. Res. 49, 5002-5006.

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Depuration of Okadaic acid (Diarrhetic Shellfish Toxin) in mussels, *Mytilus edulis* (Linnaeus), feeding on different quantities of nontoxic algae

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Abstract

Depuration of mussels contaminated by Diarrhetic Shellfish Toxins (DST) is a potential option for the shellfish industry to manage the impact of DST. Field observations have suggested that the main factor regulating the rate of depuration of DST is the quantity of nontoxic algae available for the mussels to feed upon. In this paper, the effects of the quantity of food, which mussels feed upon, on the rate of depuration of DST in *Mytilus edulis* L. was tested in a laboratory experiment. Mussels naturally contaminated by the DST okadaic acid (OA) were collected from a mussel farm located on the Swedish west coast during a bloom event. Individual mussels were placed in filtered seawater and given daily rations of a mixture of nontoxic algae as follows: no food, 0.5% and 1.5% of dry weight body mass day^{-1}. Depuration was performed over 1, 2, 4, 8, 16 or 32 days. The levels of OA decreased in all treatments with time, with an average of approximately 50% reduction after 32 days. No significant differences in content of OA among food rations were detected. In contrast to predictions, a trend towards lower levels of toxins in the mussels receiving no food compared to both food treatments was observed after 32 days of depuration. The loss of toxins in mussels that were not feeding correlated with a considerable loss in the mass of the digestive gland between 16 and 32 days. It was concluded that the rate of depuration of OA in mussels is not positively correlated with digestive activity and fecal production. Instead, the lipophilic character of the OA molecule suggests that OA may have affinity for lipid-rich cellular and intracellular components. Increased usage of lipid stores, which occur during starvation, may accelerate the release of OA. This model could explain the observations made during the last part of this experiment. In management of toxic

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mussels, depuration in waters free of toxic algae is not likely to be enhanced by increasing the food supply to mussels; however, long periods of depuration in the absence of food should be avoided because of the negative effects on the condition of the mussels.

Keywords: Mussels; Diarrhetic Shellfish Toxins; Okadaic acid; Depuration; Digestive activity; Management

1. Introduction

Diarrhetic shellfish poisoning (DSP) is one of several seafood poisoning syndromes caused by marine phycotoxins. Although not a life-threatening condition, DSP is a worldwide problem for the bivalve aquaculture and fisheries industries (Hallegraeff, 1993). Closure of shellfish harvest areas for long periods due to the presence of DSP toxins (DST) are common and are causing major economical losses in some countries. For example, farmed blue mussels, *Mytilus edulis*, along the Swedish west coast generally contain levels of DST above the tolerance limit for harvest of shellfish for up to 6 months each year (Lindegarth, 1997). This is the largest impediment to the development of mussel farming in Sweden (Kollberg, 1999).

The toxic compounds mainly responsible for DSP are okadaic acid (OA) and the structurally related DTX-1, DTX-2 and DTX-3 (Yasumoto et al., 1985; Kumagai et al., 1986; Carmody et al., 1996). Except for DTX-3, which is believed to be a metabolic product in the bivalves (Lee et al., 1989), these lipophilic polyether fatty acids are produced by marine dinoflagellates of the genera *Dinophysis* and *Prorocentrum* (Yasumoto et al., 1980; Murakami et al., 1982; Murata et al., 1982). The toxicity of the OA group of compounds comes from their ability to bind and inhibit the activity of protein phosphatases, which in turn causes major effects on signal transduction pathways inside cells (Bialojan and Takai, 1988; Haystead et al., 1989).

Different options for how the negative impacts of DST can be managed and minimised have been proposed (Christophersen and Strand, 1994; Dijkema et al., 1995; Blanco et al., 1999; Kollberg, 1999). In several areas, DST-producing algae have been reported to be more or less permanently absent (Sedmak and Fanuko, 1991; Haamer, 1995; Poletti et al., 1996). Such waters, usually fjords, bays and lagoons characterized by restricted inflow of water from the open sea, can be used permanently for farming but also temporarily for ‘storing’ and depuration of bivalves, which are relocated from other areas.

Another option is to develop methods for the depuration of toxic mussels in natural or controlled systems. However, to achieve cost-effective depuration both in controlled systems and in the natural environment, it is essential to identify whether environmental conditions affect the depuration kinetics of DST. Also important is to understand the physiological mechanisms by which bivalves eliminate DST.

Some field observations and experiments on depuration of DST in various species of bivalves have been performed (Lindahl and Hageltorn, 1986; Haamer et al., 1990; Sampayo et al., 1990; Marcaillou-Le Baut et al., 1993; Sedmak, 1995; Bauder et al., 1996; Poletti et al., 1996; Blanco et al., 1999). Some of these studies have suggested

that the quantity of nontoxic food resources is the most important factor regulating the rate of depuration. One physiological model to explain how food affects the rate of depuration of DST in mussels is discussed in Blanco et al. (1999). When nontoxic food resources become more abundant, ingestion rates in the mussels increase, which in turn leads to a higher digestive activity and greater metabolic fecal loss. Fecal deposition has been suggested to be the main route for elimination of DST (Bauder et al., 1996; Blanco et al., 1999). Thus, as a consequence of increased feeding rates, it is hypothesized that DST is eliminated at a higher rate in the mussels. In Swedish blue mussels, high levels of OA are generally detected during the autumn and winter period, followed by a fast reduction during early spring. The reduction of OA coincides with the spring bloom of diatoms. This indicates that feeding on nontoxic algae may be important for depuration. The observations of positive correlations between the concentration of algae and the rate of depuration is consistent with, but not evidence for the notion that high rates of ingestion causes high rates of depuration. This has yet to be shown in manipulative experiments.

Here, the model that the supply of nontoxic algae to mussels affects the rate of depuration is tested. It was predicted that the concentration of OA would decrease at a greater rate in mussels, which received larger concentrations of food than in those receiving lower concentrations or no food. A laboratory experiment was performed where blue mussels, naturally contaminated by OA, were supplied with different rations of algae and depurated for up to 32 days.

2. Materials and methods

2.1. Animals and algal food source

Blue mussels containing OA were collected from a long-line mussel cultivation farm on the 23rd of October, 1998, 1 day prior to the start of the experiment. The farm is situated in the vicinity of Tjärnö Marine Biological Station on the west coast of Sweden where the experiment was conducted. Levels of OA above the limit for harvest (160 μg kg^{-1} mussel meat) had been detected for approximately 1 month prior to October 23. Monitoring data from the farm between July 1998 and April 1999 is shown in Fig. 1 and the experimental period is indicated in the graph. Equally sized mussels (age 3–5 years, shell length 85 ± 3.5 mm S.D.) were selected for the experiment and a subsample was taken for determination of soft tissue dry weight (4.6 ± 1.5 g) and OA (2.90 ± 0.90 μg OA g^{-1} digestive gland). The mussels were kept in air at 4 °C overnight.

A mixture of two species of unicelled algae were used as food: *Isochrysis galbana* var. *tahaitian* (T-ISO), and *Thalassiosira pseudonana* (3H), supplied as algal pastes from Reed Mariculture, Inland Sea Farm, USA. The dry mass per volume unit (mg ml^{-1}) was estimated for each paste and based on this, equal amounts of paste were then combined in the proportions four parts of T-ISO to five parts of 3H. The final cell concentration was 5.6×10^9 cells g^{-1} dry mass. The algal pastes were resuspended in filtered seawater by a magnetic stirrer to desired concentrations.

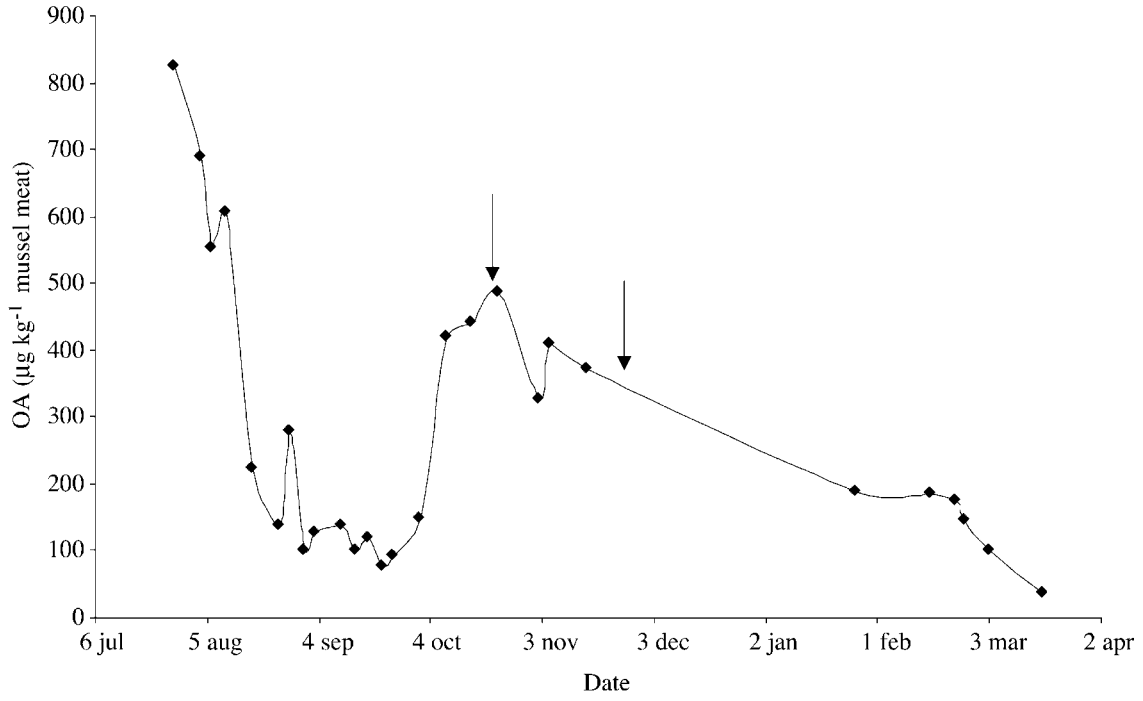

Fig. 1. Data from the national monitoring program on concentration of OA (µg kg^{-1} mussel meat) in mussels from Tjärnö Vattenbruk farm site from July 1998 to April 1999. The experimental period is indicated between the arrows.

2.2. Experimental conditions

The experiment was designed as a two-factor analysis of variance (ANOVA) with food rations and days of depuration as fixed factors. Three levels of food rations were used: no food, 0.5% (ration 1) and 1.5% (ration 2) of dry body mass day^{-1}. The total amount of algae and cell numbers in each food ration is shown in Table 1. These food rations were chosen as moderate and high concentrations and were based on observations on feeding rates for mussels of the size employed in this experiment (Bayne, 1976).

The mussels were depurated for 1, 2, 4, 8, 16 or 32 days. The animals were then sacrificed for subsequent weight measurements and analysis of OA content. Five replicate mussels were included per treatment at each time. Thus, a total of 90 mussels were included in the experiment.

The experiment was conducted between October 24 and November 26, 1998 at 10 °C. This corresponded to the water temperature in the field when mussels were collected.

Table 1
Total amounts of algae in food rations 1 and 2

	A	B	C
Ration 1	0.5	23	129
Ration 2	1.5	69	387

(A) % dry body mass day^{-1}.
(B) mg dry weight day^{-1}.
(C) cell numbers $\times 10^6$ day^{-1}.

Individual mussels were placed in 1l plastic beakers containing 800 ml of filtered natural seawater (0.2 μm Millipore filter) with a salinity of 2.5%. Air was supplemented to each beaker in order to keep the algae in suspension and to avoid oxygen depletion, as well as to reduce ammonia levels. Care was taken not to resuspend any fecal material. Every second day during the experiment, the whole volume of water was replaced by freshly filtered seawater. Prior to changing water, the fecal deposits were collected by a Pasteur pipette into preweighed glass vials and stored in a freezer for determination of total fecal production for each depuration period. The insides of the beakers were cleaned to avoid microbial growth.

Algal particles were added by pipetting 5 ml algal suspension to each beaker 10 times per day. Before adding new algae, the water was visually inspected to verify that the mussels had cleared the cells from the previous addition. To the no-food treatment, 5 ml of filtered seawater was added at each feeding occasion. Ingestion rates were not explicitly measured but the production of faeces was measured and used as an indirect measure of ingestion. Only minor amounts of pseudofaeces were occasionally produced in the high ration level. This was not included in the estimation of fecal production.

2.3. Measurements

When mussels were sacrificed, soft tissue was removed from the shell and drained on paper whereafter the digestive gland was separated from the rest of the tissue. Total soft tissue wet weights and weights of the digestive glands were determined. The digestive glands were then immediately frozen in -20 °C for chemical analysis of OA.

Content of OA and DTX-1 was analyzed in individual digestive glands according to the HPLC method of Lee et al. (1987) with minor modifications. The 1-pyrenyldiazomethan (PDAM, from Molecular Probes, Europe, the Netherlands) was used as a fluorescent labelling agent and for clean-up procedures, silica gel cartridge columns were used.

Faeces produced for each treatment were stored in -20 °C and dry mass was later determined by incubating the glass vials for 12 h in 80 °C. OA content of faeces was not measured.

2.4. Statistical analysis

Differences among treatments were analysed using a two-factor analysis of variance (ANOVA). Data was first checked for homogeneity of variances using Cochran's test. For significant effects, Student–Neuman–Keuls (SNK) a posteriori test for differences among means was applied.

3. Results

3.1. Concentration of OA in the digestive gland

The effects of food rations and days of depuration on concentration of OA are shown in Fig. 2a. In general, the concentration of OA was reduced in all rations

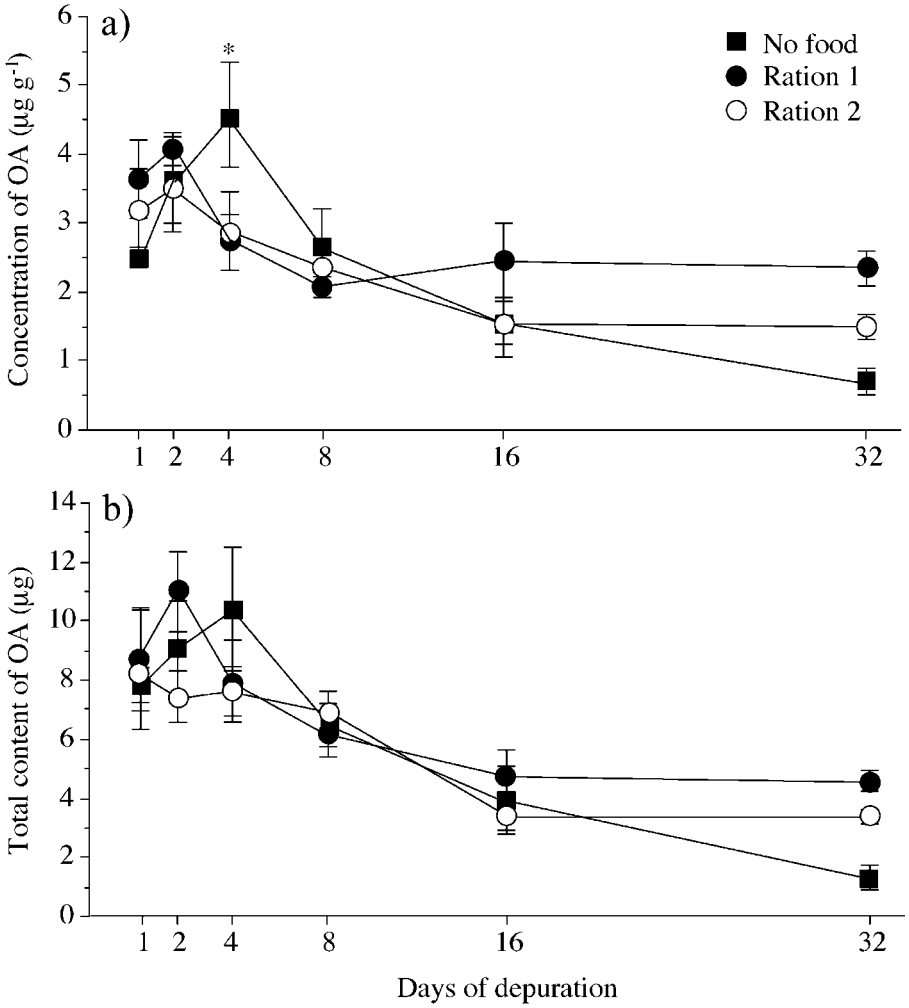

Fig. 2. The effects of food rations and days of depuration on levels of OA. (a) Concentration of OA, expressed as µg g^{-1} digestive gland. (b) Total content of OA, expressed as µg in the digestive gland. Error bars represent S.E.

during the experiment from 3.1 µg OA g^{-1} digestive gland (dg) on average at day 1 to 1.51 µg OA g^{-1} dg at day 32. Between the first 2 days, there was a trend towards an increase in the toxin concentration for all treatments. In mussels feeding on rations 1 and 2, the concentration of OA was then reduced after 4 days, whereas in the mussels receiving no food, there was a further increase. At day 4, the toxin concentration in mussels receiving no food was significantly higher compared to rations 1 and 2 ($p < 0.05$ for food ration × depuration days, Table 2). This was the only day where a significant difference was observed between treatments. Although not statistically different, the second largest effect among experimental treatments was observed after 32 days of depuration. At this time, the lowest concentration of OA was observed in mussels receiving no food. This result was qualitatively contradictory to the predicted effects of food concentration, since it seemed to indicate that mussels receiving no food might have a faster reduction of OA.

Table 2
ANOVA on the effects of food ration and days of depuration on concentration of okadaic acid content (μg OA g^{-1} digestive gland), total content of OA in individual digestive glands (μg OA) and fecal production (g dry weight), untransformed data

Source of variation	*df*	Concentration of OA			Total content of OA			Fecal production		
		MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Food Ration	2	1.32	1.28	0.28	7.46	1.11	0.34	0.10	244.1	0.0001
Days	5	11.96	11.62	0.0001	98.86	14.69	**0.0001**	0.09	212.9	0.0001
Ration × Days	10	2.28	2.21	**0.03**	7.46	1.11	0.37	0.03	65.4	**0.0001**
Residual	72	1.03			6.73			0.0004		
SNK		day 4: no food>ration 2 = ration 1			day 2 = day 4 = day 1 = day 8>day 16 = day 32			day 8, 16 and 32: no food < ration 1 < ration 2		

For significant effects ($p < 0.05$), Student–Neuman–Keuls (SNK) a posteriori test for differences among means was applied.

3.2. Total content of OA in the digestive gland

The increase in the concentration of OA during the first days of depuration was somewhat confusing because no toxin-producing algae are added to the mussels. This increase can be explained if the digestive gland mass is reduced and at the same time, the content of OA remains constant or decreases at a lower rate compared to digestive gland mass. Because concentration of OA is calculated as micrograms per gram digestive gland, this could then render higher concentrations of OA. This explanation is supported by the observations of the weights of the digestive glands (Table 3). The increased concentration of OA at day 4 in the mussels, which were starved, coincides with a lower weight of the digestive glands compared to the other food treatments. Therefore, an additional analysis was done on the total content of OA (concentration of OA multiplied by the mass of the digestive gland), which is illustrated in Fig. 2b. The mean content of OA was reduced in all treatments from 8.3 μg on day 1 to 3.1 μg OA on day 32. Toxin content was significantly

Table 3
Soft tissue wet weights and digestive glands wet weights for each food ration and depuration period during the experiment

	Days of depuration					
	1	2	4	8	16	32
Soft tissue wet weight (g)						
No food	18.2 ± 1.6	16.8 ± 4.0	15.7 ± 4.2	16.5 ± 1.7	17.3 ± 3.7	14.4 ± 3.1
Ration 1	16.6 ± 1.9	16.5 ± 3.6	18.9 ± 1.4	19.8 ± 2.2	13.5 ± 2.5	15.5 ± 2.9
Ration 2	17.7 ± 2.3	17.5 ± 3.4	15.7 ± 4.9	19.2 ± 2.2	15.8 ± 1.0	16.6 ± 1.8
Digestive gland wet weight (g)						
No food	3.1 ± 0.5	2.5 ± 0.8	2.2 ± 0.5	2.6 ± 0.6	3.0 ± 0.5	1.7 ± 0.4
Ration 1	2.3 ± 0.2	2.7 ± 0.5	2.9 ± 0.4	2.9 ± 0.4	2.0 ± 0.6	2.0 ± 0.3
Ration 2	2.6 ± 0.5	2.3 ± 0.6	2.9 ± 1.0	3.0 ± 0.5	2.3 ± 0.4	2.4 ± 0.5

Values are means ± S.D. ($n = 5$).

lower after 16 days of depuration and thereafter ($p < 0.0001$, Table 2). No significant differences between mussels fed on the different food rations after 4 days of depuration were detected, which indicated that reduction in the mass of the digestive glands in mussels receiving no food contributed to the significant increase in the concentration of OA in the previous analysis. Even so, a trend towards an increase in content of OA for both ration 1 and the no-food treatment during the first days of depuration remained. The difference, although not statistically significant, between mussels receiving no food on one hand and mussels feeding on rations 1 and 2 was further pronounced after 32 days. Mean total content in the mussels, which did not receive any food, was 1.3 μg OA compared to 4.6 and 3.4 μg OA in rations 1 and 2, respectively.

3.3. Fecal production

Total fecal production (g dry weight) for each treatment during the experiment is shown in Fig. 3. The amount of faeces produced differed significantly among treatments after 8 days and thereafter (Table 2). Ration 2 had a higher production compared to ration 1, which in turn produced more faeces, compared to the starved mussels. In the no-food treatment, only a minor increase in total fecal production was observed during the whole experiment, indicating that no further ingestion occurred. The small increase in fecal production towards the end of the experiment is probably due to metabolic fecal loss. Overall, the differences in fecal production among treatments indirectly confirmed that mussels were feeding and ingesting food according to what was intended in the experimental design.

3.4. Soft tissue and digestive gland mass

Average mass of soft tissues and digestive glands for each treatment during the experiment are presented in Table 3. Some temporal variability among treatments was

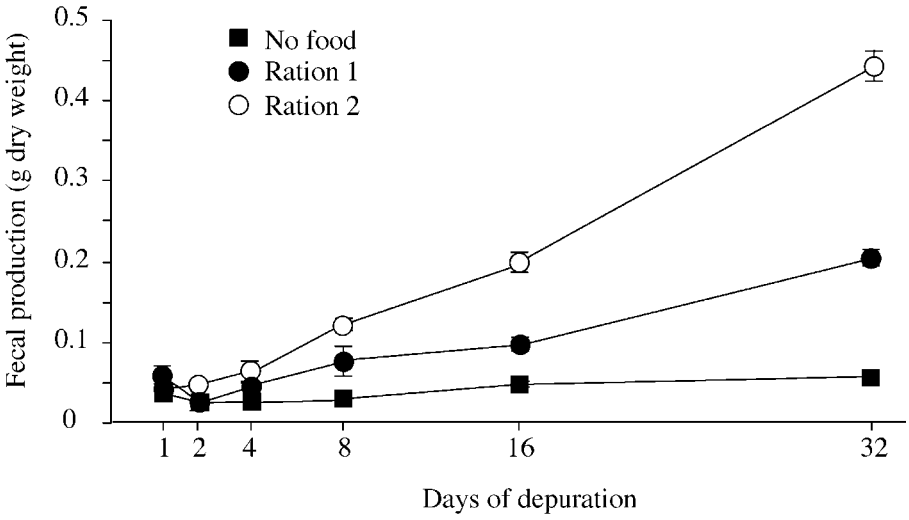

Fig. 3. Total fecal production for each treatment, expressed as g dry weight. Error bars represent S.E.

observed. In general, the temporal changes for mussels feeding on rations 1 and 2 were very similar. For example, the mass of soft tissue and digestive gland seemed to increase slightly from day 1 to day 8 and then decrease between 8 and 16 days of depuration. After 32 days, tissue and digestive gland mass were more or less similar to the initial values at day 1. For mussels that received no food, a trend towards a decrease in tissue and digestive gland mass was observed during the first 4 days and was followed by a slight increase up to 16 days. Both tissue and digestive gland mass then decreased again and was lower at day 32 compared to day 1. The tissue was also visually inspected when the mussels were dissected. Mussels that were feeding on rations 1 and 2 appeared to have healthy tissues and digestive glands with a dark colour and firm texture throughout the experiment. Mussels receiving no food were showing signs of starvation, such as pale flesh colour and digestive glands as well as water-filled, soft tissues. This was most pronounced at the end of the experiment. Care should always be taken when interpreting data on tissue wet weights since differences in water content in the tissue could mask real changes in tissue mass. For starved mussels, the loss in body mass was probably larger than what was indicated by measuring wet weights. To summarise, the overall observations on total tissue and digestive gland mass indicated that mussels receiving no food behaved differently to those feeding on nontoxic algae.

4. Discussion

The availability of nontoxic food has been proposed by several authors as the main factor affecting depuration of DST in mussels (Haamer et al., 1990; Sampayo et al., 1990; Marcaillou-Le Baut et al., 1993; Poletti et al., 1996; Blanco et al., 1999). A physiological mechanism to explain how food affects rate of depuration of DST was proposed by Blanco et al. (1999) where increased ingestion rates leads to a higher digestive activity and greater metabolic fecal loss. Since fecal deposition has been suggested to be the main route for elimination of DST by Blanco et al. (1999) and Bauder et al. (1996), the toxins are eliminated at a higher rate during high ingestive activity. Field depuration by relocating mussels from toxic to nontoxic environments has been performed by Haamer et al. (1990), Marcaillou-Le Baut et al. (1993), Poletti et al. (1996) and Blanco et al. (1999). Blanco et al. (1999) found in a multivariate analysis of the effects of environmental conditions that indirect measures of particle concentration (fluorescence and light transmission) appeared to increase the rate of depuration. Marcaillou-Le Baut et al. (1993) transplanted toxic mussels to an oyster culture pond and compared depuration with mussels depurated in the laboratory. They assumed that the faster rate observed for mussels in the oyster pond was due to a higher supply of natural phytoplankton compared to the laboratory condition. However, differences in several other factors between the oyster pond and the laboratory environment may also have contributed to this result. Both Haamer et al. (1990) and Poletti et al. (1996) observed reduction in DST content when mussels were transplanted to less toxic environments; however, no measures of food content in the water was performed. Depuration experiments in the laboratory has also been done by Haamer et al. (1990) and Croci et al. (1994). Haamer et al. (1990) compared depuration of mussels in basins with or without the addition of yeast particles. Toxin content was reduced after 1

week in both treatments but the authors observed more variability among replicates in mussels not receiving yeast. Croci et al. (1994) observed a high rate of depuration in two out of three samples in ozonised artificial seawater without any food added. These two studies suggest that depuration can occur even though no food particles are added to the system.

Even though most of the observations made by these authors suggest that food availability affects the rate of depuration of DST in mussels, the results are inconclusive and lack of replicates and controls for the effects of other factors indicate that it is still not clear whether this factor affects depuration of these toxins. Thus, the experiment presented in this paper was conducted in order to test the effects of food on depuration during controlled conditions. It was found that mussels that were fed with a high ration of algae had the highest amount of fecal production compared to mussels fed with a lower ration and starving animals. This confirmed that ingestion rates differed among treatments according to the predictions and in this way the experiment was successful. However, there was no significant difference in content of OA among food treatments over time. Depuration was achieved in all treatments with an average of 50% reduction after 32 days. Somewhat faster rates of depuration have been found by Marcaillou-Le Baut et al. (1993), Poletti et al. (1996) and Blanco et al. (1999). In these studies, depuration was observed in the field where the naturally occurring seston was available for the mussels to feed upon. The laboratory environment is likely to be less favourable and more stressful for mussels, which may have influenced the overall performance and rendered the relatively lower rates of depuration. Also, only two species of algae were used and the nutritional value of these species might not have been adequate for the physiological needs of the mussels. This imply that not only the quantity of food but the composition and phytoplankton species present in the food may be important aspects to consider for rate measurements in mussels.

Visual observations during the experiment in combination with measures of fecal production did, however, confirm that the mussels were filtering and ingesting algae, indicating that the water quality was acceptable for the mussels. Also, recontamination was avoided by removing fecal pellets together with water changes continuously during the experiment. The different rates of depuration observed in other studies could be explained by seasonal variations in the physiological status of mussels. Particularly in temperate latitudes, mussels undergo annual reproductive cycles that are associated with marked seasonal changes in biological composition and physiological rate processes (Hawkins and Bayne, 1992). Since the experiment reported here was performed in November at a low temperature, it is likely that general physiological rates were slow in the mussels, which resulted in the relatively low rate of depuration.

The significant increase in the concentration of OA for mussels receiving no food compared to the other treatments after 4 days of depuration was abolished by correcting for the decrease in digestive gland mass. Similar results where toxin concentration has been found to increase rather than decrease during the first days of depuration were observed by Haamer et al. (1990) and Marcaillou-Le Baut et al. (1993), who suggested that this phenomenon might be due to transport stress causing an increased metabolic rate and reduction of digestive gland. Hence, for the interpretation of the results in depuration experiments, it is important to monitor such changes in tissue mass. Even so, a trend towards increased levels of OA for ration 1 and the no-food treatment remained after

correction for changes in digestive gland mass. High variability in content of DST among individuals of naturally contaminated mussels (Edebo et al., 1988) may contribute to the variability observed during the first days of depuration where levels seem to fluctuate in different directions between days. It is also possible that hydrolysis of esterified OA to OA by esterases in the mussel digestive gland contributed to the initial rise in OA. Recent papers have indicated that mussels are capable of transforming OA, DTX-1 and DTX-2 to 7-*O*-acyl ester derivatives (so-called DTX-3) by attaching a fatty acyl group to the corresponding parent toxin (Marr et al., 1992; Fernández et al., 1996,1998; Suzuki and Mitsuya, 2001). The esterified DSTs exhibit low polarity and are not detected in standard HPLC analysis because a hydrolysis step to convert the esterified DST to its respective parent compound must be included in the analysis. Hence, a bioconversion of esterified OA to OA in the mussel tissue might have occurred, which could explain the increased, although not statistically different levels of OA during the first days of depuration. Biotransformation of OA to 7-*O*-acyl OA is likely to be a route for detoxification of OA in mussels since esterified OA exhibits lower toxicity, i.e. it is a less potent inhibitor of protein phosphatases (Yasumoto et al., 1989).

It was observed that the rate of depuration for feeding mussels was faster during the first 2 weeks and then slowed down during the last part, indicating biphasic depuration kinetics. This suggests that the toxins are distributed into two compartments with different depuration kinetics. Similar patterns have also been observed by Marcaillou-Le Baut et al. (1993), Fernández et al. (1998) and Blanco et al. (1999) for blue mussels and also for scallops by Bauder et al. (1996). Bauder et al. (1996) found that the rapid loss of toxins during the first 3 days of depuration coincided with the evacuation of toxin-producing algae from the viscera. Fecal deposition of recently ingested toxic algae and toxin not already incorporated into the tissue may hence be important for the release of DST during the first days of depuration. It seems possible that this process could be increased by adding food to the system. This was also observed in our experiment where content of OA in mussels feeding on the high ration was slightly lower on day 2 compared to the other treatments.

A pool of toxins more tightly bound to the tissue also seem to exist. The distribution and affinity of DST in the digestive gland tissue is likely to be governed to some degree by the physicochemical properties of the toxins. Following the pattern observed for xenobiotics such as organic contaminants, the degree of bioaccumulation depends mainly on the hydrophobicity of the contaminant together with the lipid content of the organism (Phillips, 1993). Similar pattern may be predicted for DST. The DST is lipophilic (Yasumoto et al., 1978) and the digestive gland is a major site for lipid storage in mussels (De Zwaan and Mathieu, 1992). The DST may thus have affinity for lipid-rich cellular and intracellular components such as lipid droplets or membrane structures. The release of DST from this compartment would then be dependent on a turnover of such cellular components. The route for elimination of DST from this compartment is probably through metabolic fecal loss as suggested by Blanco et al. (1999) but increasing the digestive activity by feeding the mussels may not result in a higher loss of DST. Instead, increased usage of lipid storages, which occur during late stages of starvation, might enhance the rate of depuration. This model could explain our observation that mussels given no food had a tendency to loose more toxins at the end of the experiment compared to the feeding

mussels. In future experiments, the relationships between lipid content and levels of toxin during depuration should be explored. To understand the depuration mechanisms, it is also important to identify more precisely where in the digestive gland tissue the DST are localised during uptake and depuration.

Studies on the effects of food on depuration of other types of toxins and contaminants in mussels have been investigated. Novaczek et al. (1992) and Wohlgeschaffen et al. (1992) found no difference in depuration rate of the hydrophilic neurotoxin domoic acid in feeding compared to starving *M. edulis*. Multivariate analysis of the effects of environmental factors on depuration of paralytic shellfish toxins (PST) in *Mytilus galloprovincialis* by Blanco et al. (1997) found that environmental variables, including phytoplankton concentration, seemed to be unimportant for the detoxification process. Chen and Chou (2001) observed that the depuration efficiency of PST in the purple clam, *Hiatula rostrata*, was similar for clams fed with nontoxic algae and starvation. Depuration of three congeners of hydrophobic polychlorinated biphenyls (PCB) in blue mussels was not affected by different algal concentrations (Björk and Gilek, 1997). In summary, depuration of various exogenous substances, including OA in our experiment, seems to be unaffected by food availability and occur regardless of whether the mussels are feeding or not.

In the natural field situation, depuration is likely to occur in mussels when the ingestion of toxic algae per time unit is reduced. This happens if the concentration of toxic algae decreases or disappears from the water column but filtration is maintained at a constant rate in the mussels. Also, an increase in the relative abundance of nontoxic species accompanying *Dinophysis* spp. may enhance depuration of DST. This was observed by Sampayo et al. (1990) in Portuguese waters. They suggested that a high relative abundance of nontoxic phytoplankton caused reduction in the filtration activities of the mussels in order to regulate their physiological needs for food. The intake of *Dinophysis* per time unit would thus decrease. This model is supported by several studies reviewed by Hawkins and Bayne (1992), where mussels have been found to maintain relatively constant rates of nutrient acquisition by adjusting rates of ingestion against their needs for maintenance and growth. This could also explain the increased rates of depuration of DST in mussels in Sweden during the onset of the spring bloom of diatoms. Usually, *Dinophysis* is still present in the water column during this period. This model together with the results from this paper suggest that the quantity of nontoxic food affects the mechanism of accumulation of DST rather than directly affecting the mechanism of depuration. Similar results concerning the rate of accumulation of PST in *M. galloprovincialis* was published by Morono et al. (2001) who found that accumulation of toxins was dependent on the volume-specific toxin concentration (VOSTOC) in a mixture of food particles.

5. Conclusions

Depuration can be an alternative for management of toxic mussels in order to ensure a continuous supply of mussels to the market. The results from this study have shown that depuration occurs even without the presence of algae in the system and that levels of OA can be reduced to approximately 50% of initial toxin content within 16 days. Depuration systems for mussels containing DST could hence be developed without the addition of

extra food, which reduces the cost for such systems. Long periods of depuration during conditions where no food is available is, however, not recommended since it has negative effects on the condition of the mussels, resulting in a loss of the commercial value. For depuration to be successful, i.e. to reach levels below the limit for marketing of mussels, it should be started before the mussels have accumulated high levels of toxin since depuration seems more effective during the first weeks. In order to optimise the conditions for depuration of DST in mussels, more information about seasonal variability as well as the effects of other environmental factors such as salinity and temperature on the rate of depuration is needed. The influence of phytoplankton composition in the food should also be of interest in future studies. These should also include the analysis of the DST ester derivatives and take into account their additional toxicity as well as evaluating the importance of biotransformation for detoxification of DST in mussels.

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References

Bauder, A.G., Cembella, A.D., Quilliam, M.A., 1996. Dynamics of Diarrhetic Shellfish Toxins from the dinoflagellate *Prorocentrum lima*, in the bay scallop, *Argopecten irradians*. In: Yasumoto, T., Oshima, Y. (Eds.), Harmful and Toxic Algal Blooms. Elsevier, Amsterdam, pp. 433–436.

Bayne, B.L., 1976. Marine mussels their ecology and physiology. International Biological Programme, vol. 10. Cambridge University Press, Cambridge.

Bialojan, C., Takai, A., 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Biochem. J. 256, 283–290.

Björk, M., Gilek, M., 1997. Bioaccumulation kinetics of PCB 31, 49 and 153 in the blue mussel, *Mytilus edulis* L. as a function of algal food concentration. Aquat. Toxicol. 38, 101–123.

Blanco, J., Morono, A., Franco, J., Reyero, M.I., 1997. PSP detoxification kinetics in the mussel *Mytilus galloprovincialis*. One- and two-compartment models and the effect of some environmental variables. Mar. Ecol., Prog. Ser. 158, 165–175.

Blanco, J., Fernández, M.L., Míguez, A., Morono, A., 1999. Okadaic acid depuration in the mussel *Mytilus galloprovincialis*: one- and two-compartment models and the effect of environmental conditions. Mar. Ecol., Prog. Ser. 176, 153–163.

Carmody, E.P., James, K.J., Kelly, S.S., 1996. Dinophysistoxin-2: the predominant diarrhoetic shellfish toxin in Ireland. Toxicon 34 (3), 351–359.

Chen, C.Y., Chou, H.N., 2001. Accumulation and depuration of paralytic shellfish poisoning toxins by purple clam *Hiatula rostrata* Lightoot. Toxicon 39, 1029–1034.

Christophersen, G., Strand, Ö., 1994. Algetoksiner i blåskjell. Senter for milgö-og resursstudier, Universitetet i Bergen. Report nr. SMR 17/94 (In Norwegian).

Croci, L., Toti, L., De Medici, D., Cozzi, L., 1994. Diarrhetic shellfish poisoning in mussels: comparison of methods of detection and determination of the effectiveness of depuration. Int. J. Food Microbiol. 24, 337–342.

De Zwaan, A., Mathieu, M., 1992. Cellular energy metabolism in the Mytilidae: an overview. In: Gosling, E. (Ed.), The Mussel *Mytilus*: Ecology, Physiology, Genetics and Culture. Elsevier, Amsterdam, pp. 223–307.

Dijkema R., Hagel, P., Zevenboom, W., Peperzak, L., 1995. Feasibility of Active Biological Monitoring (ABM) of phytoplankton toxins with suspended mussels (*Mytilus edulis*) in the Dutch coastal area. ICES report nr. L:29 Ref. E, K.

Edebo, L., Lange, S., Li, X.P., Allenmark, S., Lindgren, K., Thompson, R., 1988. Seasonal, geographic and individual variation of okadaic acid content in cultivated mussels in Sweden. APMIS 96, 1036–1042.

Fernández, M.L., Míguez, A., Cacho, E., Martínez, A., 1996. Detection of okadaic acid esters in the hexane extracts of Spanish mussels. Toxicon 34 (3), 381–387.

Fernández, M.L., Míguez, A., Morono, A., Cacho, E., Martínez, A., Blanco, J., 1998. Detoxification of low polarity toxins (DTX-3) from mussels *Mytilus galloprovincialis* in Spain. In: Reguera, B., Blanco, J., Fernández, M.L., Wyatt, T. (Eds.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 449–452.

Haamer, J., 1995. Presence of the phycotoxin okadaic acid in mussels (*Mytilus edulis*) in relation to nutrient composition in a swedish coastal water. J. Shellfish Res. 14 (1), 209–216.

Haamer, J., Andersson, P.-O., Lange, S., Li, X.P., Edebo, L., 1990. Effects of transplantation and reimmersion of mussels *Mytilus edulis* L. on their contents of okadaic acid. J. Shellfish Res. 9 (1), 109–112.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32 (2), 79–99.

Hawkins, A.J.S., Bayne, B.L., 1992. Physiological interrelations, and the regulation of production. In: Gosling, E. (Ed.), The Mussel Mytilus: Ecology, Physiology, Genetics and Culture. Elsevier, Amsterdam, pp. 171–222.

Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P., Hardie, D.G., 1989. Effects of the tumour-promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 337, 78–81.

Kollberg, S., 1999. Beskrivning av den svenska musselnäringen. Vattenbrukarnas Riksförbund, Göteborg. In Swedish.

Kumagai, M., Yanagi, T., Murata, M., Yasumoto, T., Kat, M., Lassus, P., Rodriguez-Vazquez, J.A., 1986. Okadaic acid as the causative toxin of diarrhetic shellfish poisoning in Europe. Agric. Biol. Chem. 50 (11), 2853–2857.

Lee, J.S., Yanagi, T., Kenma, R., Yasumoto, T., 1987. Fluorometric determination of diarrhetic shellfish toxins by high pressure liquid chromatography. Agric. Biol. Chem. 51, 877–881.

Lee, J.-S., Igarashi, T., Fraga, S., Dahl, E., Hovgaard, P., Yasumoto, T., 1989. Determination of diarrhetic shellfish toxins in various dinoflagellate species. J. Appl. Phycol. 1, 147–152.

Lindahl, O., Hageltorn, M., 1986. Detoxification experiments of DSP in blue mussels. In: Proceedings of the 4th Nordic Veterinary Congress, Stockholm, pp. 463–466.

Lindegarth, M., 1997. Sammanställning och tolkning av de kontinuerliga mätningarna av okadasyrahalter längs den svenska västkusten. Bohuskustens Vattenvårdsförbund, Göteborg. In Swedish.

Marcaillou-Le Baut, C., Bardin, B., Bardouil, M., Bohec, M., Le Dean, L., Masselin, P., Truquet, P., 1993. DSP depuration rates of mussels reared in a laboratory and an aquaculture pond. In: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, Amsterdam, pp. 531–535.

Marr, J.C., Hu, T., Pleasance, S., Quilliam, M.A., Wright, J.L.C., 1992. Detection of new 7-*O*-acyl derivatives of diarrhetic shellfish poisoning toxins by liquid chromatography-mass spectrometry. Toxicon 30 (12), 1621–1630.

Morono, A., Franco, J., Miranda, M., Reyero., M.I., Blanco, J., 2001. The effect of size, temperature, seston volume, food quality and volume-specific toxin concentration on the uptake rate of PSP toxins by mussels (*Mytilus galloprovincialis* Lmk). J. Exp. Mar. Biol. Ecol. 257, 117–132.

Murakami, Y., Oshima, Y., Yasumoto, T., 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. Bull. Jpn. Soc. Sci. Fish. 48, 69–72.

Murata, M., Shimatani, M., Sugitani, H., Oshima, Y., Yasumoto, T., 1982. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. Bull. Jpn. Soc. Sci. Fish. 48, 549–552.

Novaczek, I., Madhyastha, M.S., Ablett, R.F., Donald, A., Johnson, G., Nijjar, M.S., Sims, D.E., 1992. Depuration of domoic acid from live blue mussels (*Mytilus edulis*). Can. J. Fish Aquat. Sci. 49, 312–318.

Phillips, D.J.H., 1993. Bioaccumulation. In: Calow, P. (Ed.), Handbook of Ecotoxicology. Blackwell, Oxford, pp. 378–398.

Poletti, R., Viviani, R., Casadei, C., Lucentini, L., Funari, E., Draisci, R., 1996. Decontamination dynamics of mussels naturally contaminated with diarrhetic toxins relocated to a basin of the Adriatic sea. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 429–432.

Sampayo, M.A., Alvito, P., Franca, S., Sousa, I., 1990. *Dinophysis* spp. toxicity and relation to accompanying species. In: Granéli, E., Sundström, B., Edler, L., Anderson, D.M.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 215–220.

Sedmak, B., 1995. Toxicity of different shellfish species from the Northern Atlantic. Period. Biol. 97 (4), 365–368.

Sedmak, B., Fanuko, N., 1991. Occurrence of *Dinophysis* spp. and toxic shellfish in the Northern Adriatic. J. Appl. Phycol. 3, 289–294.

Suzuki, T., Mitsuya, T., 2001. Comparison of dinophysistoxin-1 and esterified dinophysistoxin-1 (dinophysistoxin-3) content in the scallop *Pactinopecten yessoensis* and the mussel *Mytilus galloprovincialis*. Toxicon 39, 905–908.

Wohlgeschaffen, G.D., Mann, K.H., Subba Rao, D.V., Pocklington, R., 1992. Dynamics of the phycotoxin domoic acid: accumulation and excretion in two commercially important bivalves. J. Appl. Phycol. 4, 297–310.

Yasumoto, T., Oshima, Y., Yamaguchi, M., 1978. Occurrence of a new type of shellfish poisoning in the Tohoku district. Bull. Jpn. Soc. Sci. Fish. 44 (11), 1249–1255.

Yasumoto, T., Oshima, Y., Sugawara, W., Fukuyo, Y., Oguri, H., Igarashi, T., Fujita, N., 1980. Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. Bull. Jpn. Soc. Sci. Fish. 46, 1405–1411.

Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G.K., Clardy, J., 1985. Diarrhetic shellfish toxins. Tetrahedron 41, 1019–1025.

Yasumoto, T., Murata, M., Lee, J.S., Torigoe, K., 1989. Polyether toxins produced by dinoflagellates. In: Natori, S., Hashimoto, K., Ueno, Y. (Eds.), Mycotoxins and Phycotoxins ’88. Elsevier, Amsterdam, pp. 375–382.

IV

ANALYSIS OF THE RELATIONSHIP BETWEEN LIPID CONTENT AND DEPURATION OF OKADAIC ACID (DIARRHETIC SHELLFISH TOXIN) IN MUSSELS, *MYTILUS EDULIS*: RESULTS FROM A FIELD STUDY AND A LABORATORY EXPERIMENT

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*Abstract***:** Accumulation of diarrhetic shellfish toxins (DST) is a serious problem for the culture and fishery of mussels around the world. A potential strategy to increase the availability of marketable mussels is to develop large-scale depuration methods of toxic mussels. The feasibility of depuration depends to a large extent on how fast DST can be reduced in the mussels. Therefore, knowledge about the environmental conditions and physiological processes, regulating the rate of depuration of DST, is essential. In this paper, we investigated the importance of lipid turnover for elimination of okadaic acid (OA) in a field study and a manipulative laboratory experiment. Initially, the lipid content and concurrent concentration of OA in the digestive glands of farmed blue mussels, *Mytilus edulis*, was analysed during a sampling period of six months. The concentration of OA was positively correlated with the amounts of lipids in the field. This supports a previously proposed theory that increased utilization of lipid stores may accelerate the release of this toxin.

To test this causal model, a laboratory experiment was performed during 24 days. Mussels intoxicated by OA were exposed to experimental treatments, increased water temperature and food limitation, which was predicted to increase their energy requirements and need to metabolise lipids. We found that lipid content was significantly reduced in mussels exposed to an increased water temperature (24° C) compared to ambient temperature (18°C). The amount of lipids was not affected by food limitation. Although lipid content was reduced in 24°C, the rate of depuration of OA was not enhanced by this treatment and no correlation was detected between lipids and concentration of OA. Depuration rates were very similar for all treatments and followed an exponential decrease relationship ($t_{1/2}$=8 days). Thus, the proposed model that lipid metabolism affects the mechanism of elimination of OA was rejected. Nevertheless, the observed rates of depuration provide useful information and a potential predictive tool for large-scale depuration of mussels. The difficulties to influence the rate of depuration in mussels suggest that processes, insensitive to short-term manipulation of the external environment, regulate depuration of this toxin. It is proposed that seasonal changes in the physiological status of the mussels affect depuration rates of OA.

Keywords: blue mussel, depuration, diarrhetic shellfish toxin, environmental factor, lipid, okadaic acid

1. Introduction

Okadaic acid (OA) and the structurally related Dinophysistoxins (DTX-1, DTX-2 and DTX-3) are the principal toxic compounds causing diarrhetic shellfish poisoning (DSP) in humans (Yasumoto *et al.*, 1985; Carmody *et al.*, 1996; Kumagai, 1986). The DSP toxins (DST) are lipophilic polyether molecules produced by dinoflagellates of the *Dinophysis* sp. and *Prorocentrum* sp. genera and accumulate in the digestive system of bivalves when feeding on these plankton species (Yasumoto *et al.*, 1978; Yasumoto *et al.*, 1980); Murakami *et al.*, 1982; Murata *et al.*, 1982).

DST occurs in bivalves in all parts of the world and pose a serious threath to both public health and a sustainable aquaculture industry. The toxins accumulate to high levels and usually reside in shellfish for long preiods as well as being tumor promoting agents (Fujiki & Suganuma, 1993; Shumway *et al.*, 1995; Lindegarth, 1997; Vale *et al.*, 1998; Vieites & Leira Sanmartin, 2000.

There is an increasing demand from the commercial shellfish industries for management plans to reduce the impacts of DST. Large-scale depuration of toxic mussels is a potential option to increase the availability of marketable mussels (Kollberg, 1999). Wether this will be an economically feasible option depends on the rate at which DST can be reduced to acceptable levels in mussels (Shumway *et al.*, 1995). Hence, information about the influences of environmental conditions on depuration rates of DST is of great importance. Equally important is to understand the endogenous physiological processes by which mussels eliminate these compounds.

Because of its general effect upon basal metabolic rates in poikilothermic organisms, temperature is assumed to be an exogenous factor which regulate depuration rates of toxins in mussels (see reviews by Wieser, 1973 and Hawkins & Bayne, 1992; Shumway *et al.*, 1995). Observations of mussels in the field also suggest that depuration rates are affected by the availability of non-toxic food (Haamer, 1990; Sampayo *et al.*, 1990; Marcaillou-Le Baut *et al.*, 1993; Poletti *et al.*, 1996; Blanco *et al.*, 1999). A physiological process model to explain the relationship between food and the mechanism of depuration of algal toxins was discussed by Morono *et al.* (1998) and Blanco *et al.* (1999). They suggested that food increase ingestion rates which affect depuration rates by increasing the digestive activity and metabolic fecal loss, including faster elimination of DST through fecal deposition. However, this model was not supported in a laboratory depuration experiment performed by Svensson (2003) where toxic blue mussels were fed different amounts of non-toxic algae. Instead, a strong tendency for lower levels of OA in mussels receiving no food was observed during the last part of the experiment, when the mussels were showing signs of starvation.

To explain these enhanced rates of OA loss during food limitation, an alternative physiological mechanism was proposed. The lipophilic character of the OA molecule together with the relatively high lipid content of the digestive gland (De Zwaan & Mathieu, 1992) indicates that OA may have affinity for lipid-rich cellular and intracellular components such as membranes and lipid droplets. The release of OA would then be dependent on a turnover of such cellular components. Hence, increased usage of lipid storages, which occur during stressed conditions when the demand of maintenance energy exceeds net energy gain (Thompson *et al.*, 1974), could enhance the rate of depuration. Additional support for this model is given by the fact that one of the most important factor affecting bioaccumulation of lipophilic organic pollutants are the lipid content of the organism or tissue involved. Accumulation of lipophilic compounds are directly, or indirectly via variability in lipid

levels, affected by reproductive condition and seasonality (Livingstone & Pipe, 1992; Phillips, 1993; Spacie & Hamelink, 1985; Farrington, 1989).

In this paper, the model proposed by Svensson (2003) regarding the importance of lipid turnover for depuration of DST was explored. A field study was performed during January-June 2000 in order to investigate the correlation between lipid content and DST in the natural environment. Mussels, *M. edulis*, from a long-line farm was sampled once a month and the concentration of DST together with total lipid content both in the digestive gland and the mantle was measured. We also compiled data from the national monitoring program on OA and plankton concentration in the farm site area to study the temporal relationship between depuration and blooms of diatoms during this season. Secondly, a laboratory experiment was performed in August, 2000, where mussels contaminated by OA were exposed to different temperature and food conditions and depurated for 24 days. We predicted that a rise in seawater temperature and/or in combination with starvation would alter metabolic processes and result in a greater demand for energy reserves such as lipids in mussel tissue. We then predicted that mussels with reduced levels of lipids would contain less OA, that is, there would be a positive correlation between lipid content and OA. The experiment was also performed with the purpose to evaluate the effectiveness of manipulating environmental factors for depuration of OA from a management perspective.

2. Materials and methods

2.1. Field study

The field study was conducted between January to June, 2000. Blue mussels (7-8 cm shell length) were collected once every month from a long-line mussel farm at Tjärnö in the Northern archipelago of Bohuslän on the Swedish west coast. The mussels were transported to Tjärnö Marine Biological Laboratory where they were immediately frozen and stored in –74°C until the various analysis were done. At each sampling occasion, the concentration of OA in the digestive gland together with the lipid content were analysed in 6-8 individuals as described below. To study the temporal relationship between depuration of OA and spring bloom of diatoms during this year, data from the national monitoring program on concentration of OA (measured by HPLC according to Lee *et al.*, 1987), values in µg OA*kg^{-1} mussel meat) in mussels from the farm site from January to June, 2000 was compiled together with total cell concentration of diatoms (cells*l^{-1}), sampled from 0-10 m in the nearby Kosterfjord.

2.2. Laboratory experiment

The laboratory experiment was performed during 24 days, starting on the 19th of August, 2000. Mussels were harvested from a commercial long-line mussel farm 80 km south of Tjärnö Marine Biological Laboratory where the experiment was conducted. The day of collection, mussels contained 1055 µg OA*kg^{-1} mussels (data from the national monitoring program), no other DST compounds were present. In the laboratory, mussels were cleaned from epiphytic growth and sorted to obtain mussels of similar size for the experiment (shell length 70 mm). They were then kept in air at 4° C overnight until the next day when the experiment was started. A random sample of mussels were frozen in –80°C for analyses of initial levels of OA, dry weight, glycogen and lipid contents.

A mixture of four different species of microlgae was used as food supply : *Isochrysis galbana var. tahaitian* (T-ISO), *Chaetoceros gracilis* (CHGRA), *Tetraselmis sp.* and *Thalassiosira pseudonana* (3H). The algae were obtained from Reed Mariculture Inc., Inland Sea Farm, USA, as concentrated

algal pastes. Equal volumes of each algal species were mixed and resuspended in filtered seawater to desired concentrations.

Mussels were placed in plastic tanks (20 l) connected to hoses in a flow-through system (1.2 l*min^{-1}). The experimental treatments consisted of two different temperature regimes (18°C or 24°C), with or without the addition of algal food particles (food or no food). The ambient seawater temperature in the mussel farm area was 18°C when mussels were collected which also was the temperature in the laboratory water system. Immersion heaters were used to achieve 24°C which was done by directing the incoming seawater into a large water tank containing adequate numbers of immersion heaters. The warmed water was then redirected to the appropriate experimental tanks. Water flow and temperature of the incoming water was checked daily and, if necessary, adjusted to achieve a constant temperature throughout the experiment.

Food was added to the tanks from plastic 1.5 l bottles, which were placed above the experimental setup. Hoses were connected to the bottom of the bottles from which the algal suspension of desired flow rate could be supplied into the tanks. Diluted algal paste corresponding to 4.8* 10^9 cells (or 1.0 g dry weight) was added to each food treatment every day during a period of 8 hours. Earlier experiments have shown that mussels receiving no extra food experience starvation since the concentration of food particles in the incoming seawater are negligible (Svensson, 2003). As an indirect measure of ingestion, the amount of fecal production (dry weight) was estimated. At the end of the experiment, fecal pellets from each tank were collected on a filter (100 µm) and transferred to pre-weighed vials followed by drying at 80°C over night.

For each combination of temperature and food, two replicate tanks were used, yielding a total number of 8 tanks. Forty mussels were placed in each tank. Sampling was done after 8, 16 and 24 days of depuration. At each time of sampling, two replicate samples, each consisting of three pooled individuals, were taken from each tank. Each sample was analysed for OA, glycogen and lipid content. An additional four mussels were removed for individual dry weight analysis. Thus, ten individuals were removed from the tanks at each sampling occasion. In order to keep constant densities in the tanks, removed mussels were replaced by ten new mussels, which were tagged for identification but never used for analysis. After removal, mussels were immediately frozen and stored in –74°C pending analyses. Mussels were then thawed and the digestive glands were removed. Total soft tissue and digestive gland wet weights were determined for each pooled replicate sample.

2.3. Measurements

2.3.1. Preparation of samples for analysis of OA, glycogen and lipids

The digestive glands or mantle tissue were homogenized for 30 seconds in ice-cold conditions using an Ultra-Turrax knife homogenizer. 1 g of the homogenate were diluted with 4 ml of 80% methanol for analysis of OA. Appr. 500 mg of the homogenate were transferred into Eppendorff tubes and immediately frozen in –80°C for analysis of glycogen (not done in the field study). The remaining homogenate was transferred into pre-weighed test tubes and freeze-dried for analysis of total lipid content.

2.3.2. Analysis of OA in mussel tissue

The methanol extracts were centrifuged in 3000 rpm for 10 min and then cleaned using petroleum ether and chloroform extraction following the sample clean-up protocol for HPLC described by (Lee *et al.*, 1987). The chloroform extracts were used for detection of OA by protein phosphatase inhibition assay

(PPIA) according to (Vieytes *et al.*, 1997) with slight modifications (Godhe *et al.*, 2002). The assay was performed in 96 well plates and the following buffers were used: Dilution buffer (DB): 50 mM Tris-HCl pH 7.0 containing 0.1 mM $CaCl_2$. Phosphatase assay buffer (PAB): DB containing 3 mM $NiCl_2$ and 0.4 mg*ml^{-1} BSA. PP2A buffer (PP2A): PP2A (200 Units*ml^{-1}, Upstate Biotechnology) diluted in enzyme dilution buffer was added to PAB to yield a final concentration corresponding to 0.02 Units*well^{-1}. Substrate buffer (MUP): Methyl-Umbelliferyl Phosphate (Life Technologies) was diluted in DB to yield a final concentration in the wells of 42 µM MUP. An OA standard curve was prepared by dissolving OA (LC Laboratories) in 80% methanol to a stock solution of 2 µg ml^{-1}. The stock was then further diluted in DB to concentrations ranging from 0.2 to 0.0075 µg*ml^{-1} which was found to meet the linear interval for 0.02 U PP2A per well used in the assay. Before the analysis was done, 1 ml of the mussel chloroform extract was evaporated and redissolved in 100 µl of 100% methanol and 900 µl of DB was added. This extract was further diluted in order to fit the sample into the linear part of the standard curve. To start the analysis, 35 µl of PAB were added to each well and the plate was left to stand for appr. 10 min. 10 µl of sample or standard were then added in duplicate to the wells. 35 µl of PP2A were added and the plate was incubated for 15 min. in 37°C. 120 µl of MUP were added and the plate was left in room temperature in a dark place for 1 h before fluorescence was measured in a Victor multilabel counter at excitation wavelength 385 nm and emmitance wavelength 405 nm. OA concentration in the samples were calculated from the standard curve equation and expressed as µg OA equivalents*g^{-1} digestive gland (µg OA eq.*g^{-1}). Ten randomly chosen samples were also

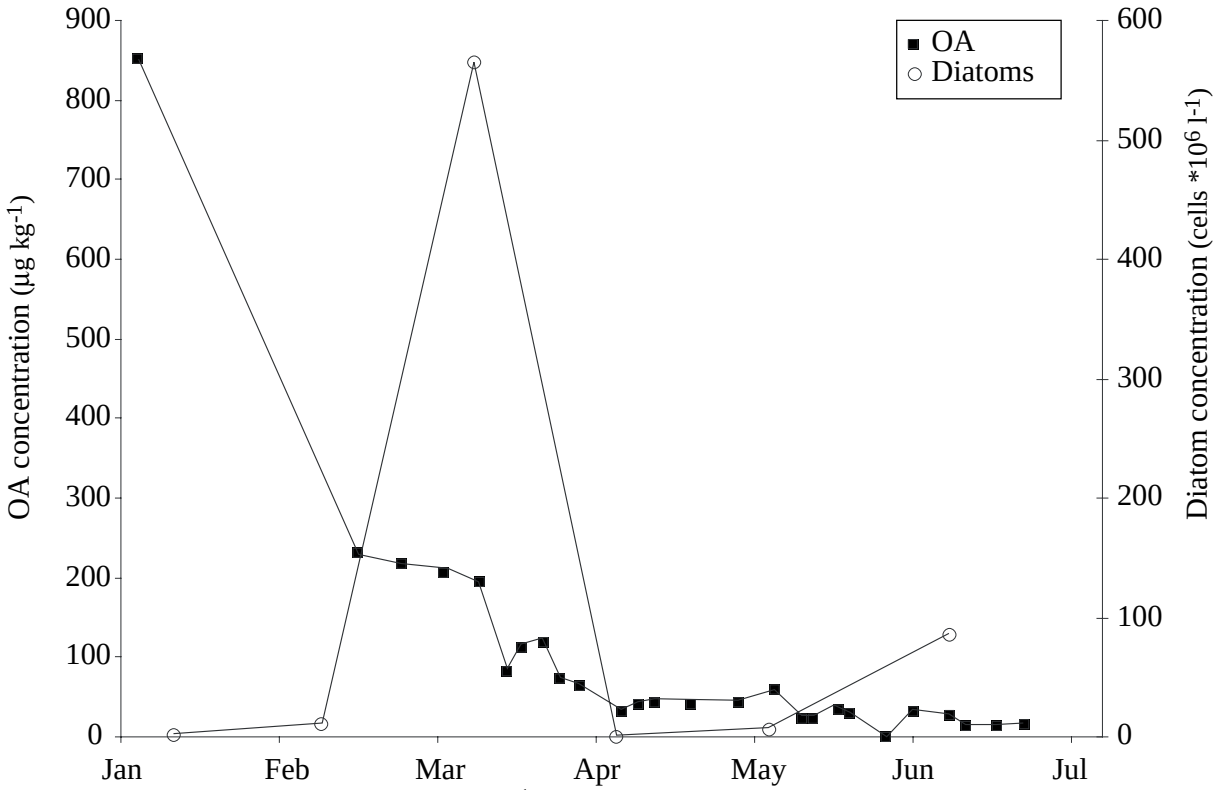

Fig. 1. OA concentration (filled squares, µg*kg^{-1} mussel meat) in mussels from Tjärnö mussel farm and total diatom concentration (open circles, cells*10^6 l^{-1} sea water, 0-10 m depth) in the Koster Archipelago between January and June 2000. Data from the national monitoring program.

analysed by HPLC (Lee *et al.*, 1987) and compared to the results from the PPIA analysis. The chemical method confirmed that OA was the only DST compound present in mussel tissue.

2.3.3. Analysis of glycogen

Concentration of glycogen in the digestive gland tissue was determined spectrophotometrically in a coupled reaction using a commercial kit (Boerhinger Mannheim, kit nr. 207748). Briefly, glycogen in the tissue is hydrolyzed into D-glucose by the enzyme amyloglucosidase. D-glucose is then phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase in the presence of ATP. Finally, G-6-P is oxidized by G-6-P dehydrogenase to D-gluconate-6-phosphate in the presence of NADH which is converted to NADPH. The amount of NADPH formed is determined by reading the absorbance at 340 nm and is stoichiometrically equivalent to the total amount of D-glucose in the tissue (free D-glucose and D-glucose formed in reaction step 1). For each sample, the amount of free D-glucose in the tissue (sample blank) was measured and subtracted from total D-glucose by omitting the enzyme amyloglucosidase in the first step of the reaction. Homogenized digestive gland tissue was diluted in ice-cold water (1:100) and used in the analysis which was performed in 96 well plates. A glycogen standard curve (0.2-2 $mg*ml^{-1}$) was used for calculation of glycogen content in samples. Glycogen concentration was expressed as $mg*g^{-1}$ wet weight.

2.3.4. Analysis of lipids

Total content of lipids in digestive gland tissue was analyzed using the gravimetric method by Gardner *et al.* (1985) with some modifications. Appr. 1 g (wet weight) of homogenized tissue was freeze-dried in pre-weighed test tubes and then finely grinded. The dry weight of the material was determined. 5 ml of chloroform-methanol (2:1) was added and the sample was thorougly mixed, ultrasonicated for 30 s. and then centrifuged for 15 min. in 3000 rpm. The supernatant was collected into new, pre-weighed test tubes and 2 ml of chloroform-methanol (2:1) was added to the remaining pellets and mixed. The solution was centrifuged again as above and the supernatant was pooled with the previous supernatant. To this extract, 1.7 ml of KCl (0.88%) was added to remove non-lipid contamination. The mixture was centrifuged in 300 rpm for 15 min and the overlying aqueous phase was removed by a Pasteur pipette. The organic solutions in the extracts were evaporated in room temperature in a fume cup-board and the test tubes were further dried in 50°C in a drying cabinet for a couple of hours. The weight of the remaining lipid fraction in the test tubes was determined and lipid content was calculated as % lipids of dry weight.

2.3.5. Statistical analysis

Analysis of variance (ANOVA) was used to test hypotheses about the effects of temperature, food and time on concentration of OA (start value not included). A three factor (fixed effects) orthogonal design was applied (Underwood, 1997). In order to control for any potential “tank effects”, the experimental unit (tank) was also replicated and included in the statistical analysis as a nested factor within temperature and food. Data was checked for homogeneity of variances using Cochran´s test and for significant effects, Student-Neuman-Keuls (SNK) a posteriori test for differences among means was applied. ANOVA was also used to test the effects of treatments on lipid content (tank not included as a factor).

Rates of depuration for each treatment was calculated by fitting the data to an exponential function, $T_t=T_0*e^{-\lambda t}$ where T_0=toxin concentration at the start of

depuration (µg OA eq.*g^{-1}), λ=exponential decay coefficient (loss*day^{-1}) and t=time (days). This assumes that the depuration rate is constant over time, which corresponds to a one-compartment model (Spacie & Hamelink, 1985; Silvert & Cembella, 1995). The correlation coefficient value (r^2) indicates how well the data fit the chosen model. Using this equation, the depuration half-lifes ($t_{1/2}$), which is the time to reach a 50% reduction in toxin content, were calculated as $t_{1/2}$=-ln(1/2)* λ^{-1}.

The relationship between lipid content and concentration of OA was tested using correlation analysis.

3. Results

3.1. Field study

Fig. 1 shows the monitoring data on OA concentration (µg*kg^{-1}) in mussels from the Tjärnö farm site and total diatom concentration (cells*10^6 l^{-1}) sampled in the nearby Koster Archipelago between January and June, 2000. OA was the only DST detected in mussels during this period. A fast decrease in toxin concentration occurred between the 4th of January (852 µg*kg^{-1}) and the 15th of February (231 µg*kg^{-1}). Low concentrations of diatoms (below 10 000 cells*l^{-1}) were observed between January to February but then a large increase occurred between 8th of February (9 400 cells*l^{-1}) and 8th of March (565 000 cells*l^{-1}). The bloom had disappeared in early April (4th of April, 56 cells*l^{-1}). This data suggest that a substantial reduction of OA in mussels occurred before the peak of the diatom spring bloom during 2000 in this area. Thus, it appears that there is no support for the model that the decrease of OA is caused by an increase in non-toxic food.

The results from the monthly sampling on OA concentration together with lipid content in the digestive gland is shown in fig. 2. Between 18th of January and 16th of February, a substantial decrease in OA concentration was observed. Mean concentration of OA in the digestive gland decreased from 3.23 to 1.78 µg*g^{-1}. This is in agreement with the data fom the monitoring program where the fastest reduction of OA was observed during the same period. OA was further decreased at each time of sampling and in June, the OA concentration was 0.31 µg*g^{-1}. Lipid content in the digestive gland showed the same trend as OA concentration between January and February with a reduction from 21.6% to 14.6%. No change in lipid content was

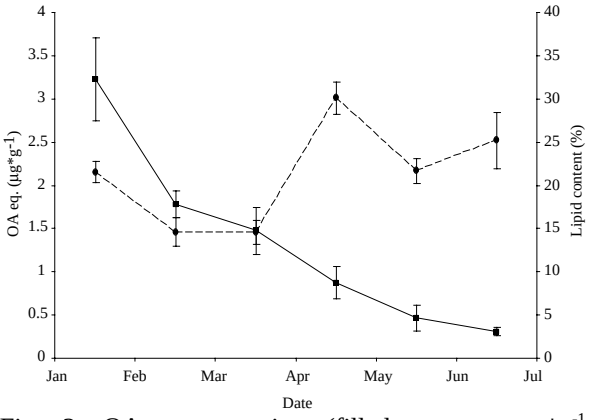

Fig. 2. OA concentration (filled squares, µg*g^{-1} digestive gland) and lipid content (% total lipids of dry weight) in the digestive gland (filled circles) in individual mussels from Tjärnö farm site between January and June, 2000. Error bars represent SE of means.

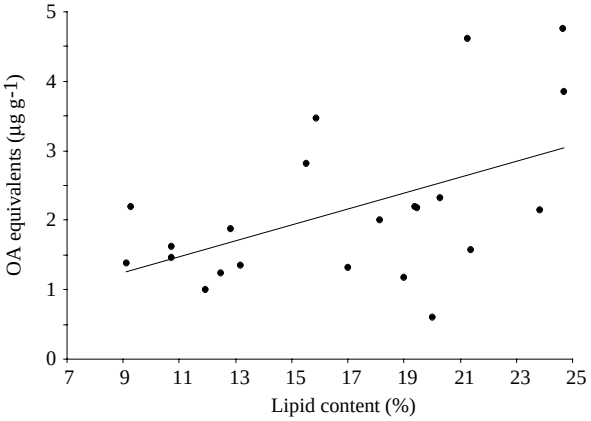

Fig. 3. Correlation between lipid content (% of dry weight) and concentration of OA (µg OA equivalents*g^{-1}) in digestive glands of individual mussels from Tjärnö farm site, data from January to March, 2000. A significant positive correlation was detected ($P<0.05$, $r^2=0.26$).

Table 1. Soft tissue dry weights (glycogen concentration in the digestive glands (mg*g^{-1} wet weight) and total lipid content in the digestive gland, expressed as % lipids of dry weight, in the various treatments. Values are means ± SD with number of replicates inside brackets.

Variable	Temperature (°C)	Food/ No food	Start	Days of depuration 8	16	24
Dry weight (g)			2.1±0.5 (8)			
	18	Food		2.0±0.4 (8)	2.1±0.8 (8)	1.9±0.9 (8)
	18	No food		1.8±0.6 (9)	1.8±0.9 (8)	2.3±0.3 (8)
	24	Food		2.1±0.7 (7)	1.6±0.5 (8)	1.2±0.7 (4)
	25	No food		2.1±0.4 (7)	2.1±0.9 (8)	1.9±0.7 (8)
Glycogen (mgg^{-1})*			42.1±31.7 (4)			
	18	Food		52.3±11.3 (4)	50.2±22.0 (4)	33.8±10.5 (4)
	18	No food		44.8±5.3 (4)	32.8±5.8 (4)	31.6±10.8 (4)
	24	Food		41.1±15.6 (4)	47.1±32.3 (4)	32.6±2.9 (4)
	24	No food		37.4±10.4 (4)	28.4±10.5 (4)	41.1±3.9 (4)
Lipid content (%)			23.0±1.6 (2)			
	18	Food		18.3±3.5 (3)	18.8±1.8 (4)	18.2±2.7 (2)
	18	No food		20.4±5.8 (3)	19.0±1.5 (4)	18.1±1.8(4)
	24	Food		19.6±2.1(3)	13.3±0.5 (2)	17.3±5.9 (4)
	24	No food		17.7±0.7 (3)	15.6±0.9 (3)	15.9±2.1 (4)

observed between February and March but in April, a large increase in lipid levels was measured (30.2%). Lipid content remained relatively high in the digestive gland throughout the rest of the study. The correlation between lipid content and OA concentration from January to March was analysed using correlation analysis, the results are shown in fig. 3. There was a significant positive relationship between these variables during the first three times of sampling ($p<0.05$). This result supported the model that the large reduction in OA content may be due to an increased use of lipid stores during this period.

3.2. Laboratory experiment

3.2.1. General observations and effects of environmental factors on physiological variables

Differences in ingestion between mussels fed the algal suspension and those not receiving food was confirmed by the production of faeces. Mussels supplied with food had produced approximately 5 times as much faeces compared to the no food treatment at day 24 (data not shown). During the last days of the experiment, some mortality was observed for feeding mussels kept in 24°C. Soft tissue dry weights, glycogen concentration and total lipid content in the digestive gland were measured as general markers of physiological stress (table 1). Mean dry weights did not change throughout the experiment in all treatments except in 24°C food where a decreasing trend was observed, indicating that mussels were loosing body mass in this treatment. Since this was also the treatment where some mortality occurred, mussels appeared to be highly stressed in this experimental treatment. The same pattern was not observed for glycogen where variability was large among replicates and there were no apparent difference among treatments. Temperature, however, significantly affected lipid content in mussel digestive glands (table 2, $p<0.05$). Compared to 18°C, lipid content in mussels subjected to 24°C was lower already after 8 days of depuration and throughout the rest of the experiment. The differences in lipid content appeared to be small, about 2% lower in 24°C on day 8 and 24 and 5% on day 16.

Table 2. ANOVA on the effects of temperature, food supply and days of depuration on lipid content in the digestive glands (% lipids of dry mass). Significant effects for differences among means ($p<0.05$) were analysed using Student-Neuman-Keuls (SNK) aposteriori test.

Source of variation	df	MS	F	P
Temperature, =Te	1	40.50	4.53	**0.04**
Food/no food, =F	1	0.02	0.00	0.96
Temp * Food	1	1.46	0.16	0.69
Time, =Ti	2	20.36	2.28	0.12
Te * Ti	2	12.15	1.36	0.27
Ti * F	2	4.78	0.54	0.59
Ti * Te * F	2	8.11	0.91	0.42
Residual	27	8.94		

SNK: 18°C>24°C

2% reduction (e.g. from 20% to 18%) represents a 10% loss of lipids, which is a rather large reduction. Food limitation did not affect lipid content in this experiment (table 2, $p>0.05$). Hence, increasing the temperature reduced lipid content but high temperature in combination with starvation did not further reduce lipid levels in the mussels.

3.2.2. Effects of environmental factors on rate of depuration of okadaic acid

The results from the experiment are shown in fig. 4. At the start of the experiment, the mussels contained on average 2.89±0.36 SE OA eq.*g^{-1} digestive gland. The effects of environmental factors on depuration of OA was analysed using ANOVA (table 3). No "tank" effect was found in this experiment

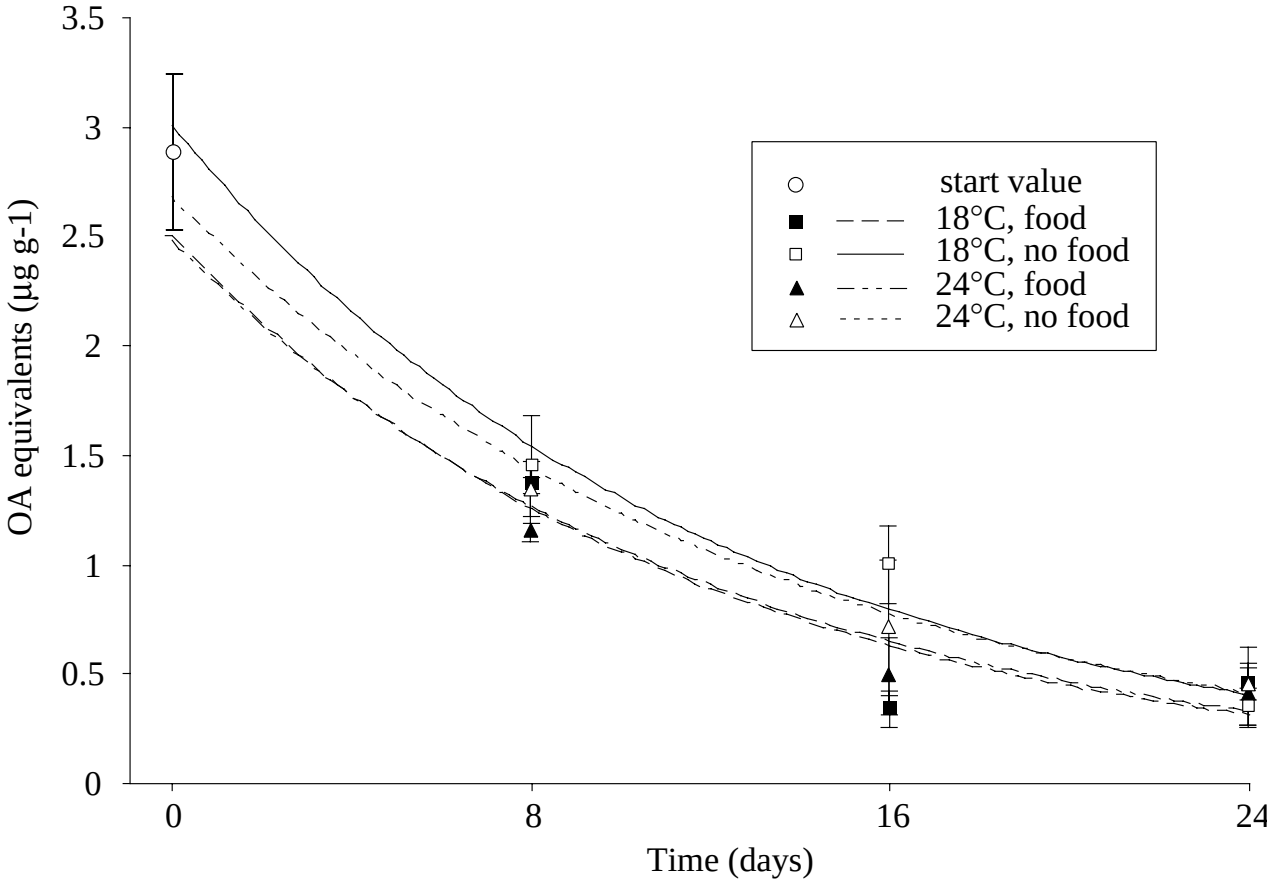

Fig. 4. The effects of temperature and food on rate of depuration of OA (OA equivalents g^{-1} digestive gland). Values are means± SE. Depuration curves for each treatment during the experiment were added by fitting data to the exponential decay equation $T_t=T_0*e^{-\lambda t}$. T_0=toxin concentration at the start of depuration (µg OA equivalents*g^{-1}), λ=exponential decay coefficient (% day^{-1}) and t=time (days). 18°C food: $T_t=2.51*e^{-0.086t}$, $r^2=0.82$; 18°C no food: $T_t=3.01*e^{-0.083t}$, $r^2=0.96$; 24°C food: $T_t=2.48*e^{-0.084t}$, $r^2=0.94$; 24°C no food: $T_t=2.68*e^{-0.077t}$, $r^2=0.99$.

(time*tank (temp, food), p=0.76), hence tests of hypotheses about effects of food, temperature, time and their interactions were done (Underwood 1998). The concentration of OA was significantly different among times (p<0.001) which was the only factor affecting concentration of OA in this experiment. Mean concentration of OA was 1.35±0.09 SE OA eq.*g^{-1} at 8 days of depuration. At 16 and 24 days, OA was reduced to 0.70±0.13 and 0.42±0.07 respectively. SNK a posteriori test showed that there were significant differences among all levels of time (8 days>16 days>24 days). Apart from the significant effect of time, there was a trend towards an interaction between time and food supply (time*food, p=0.13). Compared to the no food treatment, concentration of OA in mussels receiving food tended to be lower at both 8 days (no food: 1.44±0.17, food: 1.28±0.04) and 16 days (no food: 0.90±0.20, food: 0.46±0.12) but not at 24 days of depuration (no food: 0.40±0.10, food: 0.44±0.10). We observed that variability among replicates were larger in the no food treatment at 8 and 16 days of depuration.

To obtain rates of depuration, data from each treatment was fitted to the exponential loss equation $T_t=T_0\ e^{-\lambda t}$, fitted curves and equations are shown in fig. 4. The r^2 values ranged between 0.82-0.99, which suggested that the depuration dynamics was accurately described using a one-compartment model approach. Depuration rates were highly similar for all treatments, ranging from 8.4% day^{-1} for the 18°C food treatment to 9.3% day^{-1} for the 24°C food treatment. The average expected half-life ($t_{1/2}$) for all treatments was calculated to 8 days.

3.2.3. Relationship between lipid content and concentration of okadaic acid

The correlation between lipid content in digestive glands and OA concentration was analysed by correlation analysis (fig. 5). Data for lipid content ranged between 13 to 26% and OA concentration between 0.2 to 2.2 µg OA eq.*g^{-1}. According to the predictions, a positive correlation between these variables was expected, however, no trend towards a relationship between lipid content and OA was detected (p=0.61, r^2=0.007).

4. Discussion

Our results from the field study together with

Table 3. ANOVA on the effects of temperature, food supply and time on depuration of okadaic acid (µg OA equivalents*g^{-1} digestive gland), untransformed data. Significant interaction effects for differences among means (p<0.05) were analysed using Student-Neuman-Keuls (SNK) aposteriori test.

Source of variation	df	MS	F	P	Error Term
Temperature, =Te	1	0.06	0.71	0.45	Tank (Temp, Food)
Food/no food, =F	1	0.40	4.66	0.10	Tank (Temp, Food)
Te * F	1	0.01	0.15	0.72	Tank (Temp, Food)
Tank (Temp, Food), =Ta	4	0.08	0.82	0.52	Pooled MS
Time, =Ti	2	3.61	34.87	**0.0001**	Pooled MS
Ti * Te	2	0.04	0.36	0.70	Pooled MS
Ti * F	2	0.23	2.21	0.13	Pooled MS
Ti * Te * F	2	0.11	1.04	0.36	Pooled MS
Ti * Ta (Te * F)	8	0.07	0.61	0.76	Residual
Residual	24	0.12			
Pooled MS	32	0.10			

SNK: 8 days>16 days>24 days

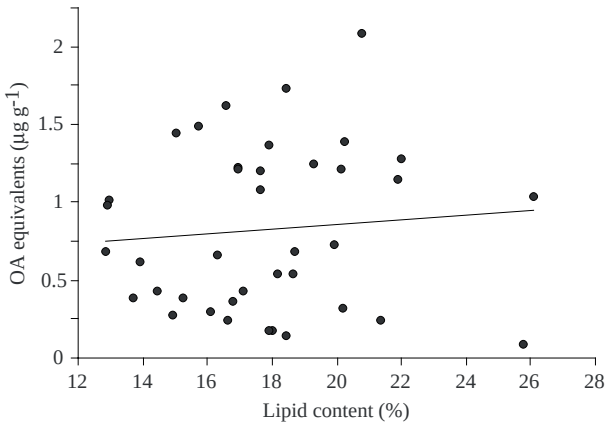

Fig. 5. Correlation between lipid content (% of dry weight) and concentration of OA (µg OA equivalents*g^{-1}) in digestive glands of mussels, data from the laboratory experiment. No correlation was detected ($P>0.05$, $r^2=0.007$).

the monitoring data from the Tjärnö region during early 2000 indicated two things: (1) a rapid decrease of OA occurred before the peak of diatoms this season (2) the reduction in OA between January to March coincided with a decrease in lipid content in the digestive glands. This supported the theory that changes in utilization of lipid stores may affect the release of lipophilic OA. The reduction in lipid content (and OA) which we observed occurred during a time of the year when food had been limited for some time which indicated that mussels depended on energy storage products such as glycogen and lipids to maintain vital processes. The digestive gland is together with the mantle the major storage organ for lipids in *Mytilus sp.* (De Zwaan & Mathieu, 1992). Lipid content has been found to remain rather constant throughout the year in *Mytilus platensis* (De Moreno *et al.*, 1980) but (Thompson *et al.*, 1974) found seasonal changes in the amount of lipids in *M. edulis* with maximum levels in July (>30% of dry weight) and minimum in late winter (11%). We found that total lipid content in the digestive glands changed considerably during this period, for example, the content doubled between March (14%) and April (30%). This increase is most likely explained by the mussels responding to the algal spring bloom, replenishing the lipid stores. Also, the increased lipid levels with concurrent decrease in OA concentration from April-June suggested that the release of this toxin is not exclusively governed by rate processes involved in metabolism of lipids.

Following on the observations of a positive correlation between lipid and OA content in the field, we wanted to test this causal model in a laboratory experiment. A prerequisite for this was to obtain variability in lipid content among individuals or groups of toxic mussels. Therefore, factors which could influence the energy requirements and need to metabolise lipids were considered. In poikilothermic organisms such as mussels, the energy requirements for maintenance metabolism are strongly regulated by the surrounding water temperature (see reviews by Wieser (1973) and Hawkins & Bayne (1992)). Transferring mussels to an immediate increase in temperature is known to result in modulation of biochemical and physiological rate processes in order to acclimate to the new condition (Hawkins *et al.*, 1987). This acclimation period may last for days or even weeks and is associated with an extra energy cost (Bayne *et al.*, 1973; Widdows & Bayne, 1971). If acclimation is incomplete, the reduction in net energy gain signifies a ‘stressed condition’ (Bayne, 1985) and material from the digestive gland is rapidly utilized during stressed conditions (Thompson *et al.*, 1974). Increased temperature is by itself associated with higher energy requirements for maintenance metabolism in poikilothermic organisms such as mussels (Wieser, 1973). Hence, we predicted that exposing mussels to an increase in temperature would increase the need to utilize lipids as an energy source compared to mussels maintained in the ambient seawater temperature. We measured this as a change in total lipid content in the digestive glands. An increase in lipid utilization was also expected for starving mussels compared to mussels receiving food. In *Mytilus* sp., metabolic rate

processes and usage of body energy reserves are highly regulated by the availability of food (Hawkins & Bayne, 1992). However, the results showed that lipid content remained unaffected by food treatment in this experiment. Bayne *et al.* (1973) and Widdows (1973) reported a decline in metabolic rates in *M. edulis* in response to starvation. Because we did not see any effects of food limitation on lipid content in our study, it seems possible that these mussels reduced the metabolic rates and energy requirements and thus need to use lipids. Alternatively, the quantity or quality of the microalgae, supplied to the mussels in the food treatment, were inadequate for maintenance, resulting in a similar response as for non-feeding mussels.

The dry weights of mussels kept in 24°C and supplied with food were reduced and mortality was observed towards the end of the experiment, indicating that mussels were highly stressed in this treatment and were using their body energy reserves for survival. This was not observed for non-feeding mussels in 24°C, which suggested that the energy requirements were lower during food limitation, possibly as a result of a general depression of metabolic rates as discussed above.

Interestingly, there was no significant effect of temperature on glycogen concentration which would be expected if glycogen reserves are used as the primary energy source before lipids as suggested by Zaba & Davies (1984). It should be noted that variability among samples was large, indicating that the difference among treatments needs to be large to detect statistical differences.

In contrast to food limitation, exposing mussels to an increased temperature reduced the amount of lipids in the digestive glands. This was in agreement with the predictions. Significantly lower levels of lipids were observed in mussels exposed to 24°C compared to 18°C at all sampling occassions. The relative differences in mean lipid content among treatments were small but variability among individual samples during the experiment ranged from 13-26%, hence we tested if OA concentration was positively correlated to the lipid content in the digestive gland. However, we found no relationship between these variables in the experiment. This was in contrast to the correlation observed in the field study. Since we could not experimentally verify the significance of this relationship, the model that lipid metabolism affects elimination of OA was not supported in the laboratory experiment.

Neither temperature nor food had any effect on the rate of depuration of OA. The rate of reduction in concentration of OA was similar for all treatments. This is in contrast to previous beliefs. It has been assumed that depuration is faster in higher temperatures (Shumway, 1995) although this was not observed in an experiment performed by Lindahl & Hageltorn (1986), who compared depuration efficiency of DST between mussels (*M. edulis*) kept in ambient temperature (0-2°C) and in heated seawater (10° C). In this paper, we exposed mussels to a instantaneous increase of 6°C and compared depuration rates with those kept in ambient seawater temperature. This approach was chosen in order to induce an increased requirement to utilize energy reserves as discussed above. A different approach to experimentally test the effects of temperature is to gradually adjust mussels to a higher temperature for some time and then compare depuration rates to mussels acclimated in lower temperature. This would abolish the stress effects and cost of acclimation and perhaps represents the “true” effects of temperature, comparable to long-term seasonal temperature changes in the sea. However, naturally intoxicated mussels cannot be used in this kind of study. We expected complete acclimation to the new temperature after 14 days in our experiment, following the results by Widdows & Bayne (1971) and Hawkins *et al.* (1987). This

suggested that any effect of temperature might have been detected after 24 days of depuration in our experiment but this was not seen. Also, feeding on non-toxic algae has been observed to increase the rate of depuration in the field but did not increase depuration rates of OA in the experiment by Svensson (2003). Our results further indicate that depuration is not accelerated in feeding mussels compared to non-feeding mussels. Alltogether, these results emphasize the difficulties to influence the mechanisms of depuration by manipulating external factors which was also the conclusion by Morono *et al.* (1998). This suggests that endogenous processes, insensitive to immediate changes in the surrounding environment, regulate the mechanism of depuration of the DST. We propose that seasonal changes in the physiological status of mussels, which may correlate to environmental factors such as food and temperature, is important in controlling depuration rates of algal toxins and may account for the variability in depuration rates observed in the field. Especially for mussel populations in temperate latitudes, the physiological status is related to the annual reproductive cycle which is associated with marked seasonal changes in both biochemical composition and physiological rate processes (Hawkins & Bayne, 1992). For example, a seasonality in the physiological response to external factors such as food have been documented for *M. edulis*. Hawkins & Bayne (1984) showed that, despite similar availability of the same algal species, rates of ingestion were less than half in a winter experiment compared to those measured during summer under identical conditions. There is also convincing evidence of an endogenous regulation of the seasonal balance between acquisition and utilization of nutrients with a considerably lower metabolic efficiency during winter compared to autumn (Hawkins, 1985; Hawkins & Bayne, 1984). It is possible that the endogenous rate processes involved in depuration of algal toxins show similar seasonal fluctuations and short-term inertia to changes in the environment.

By fitting the data to the exponential loss equation, we could calculate the rate constant for depuration of OA and depuration half-life ($t_{1/2}$). The half-life was approximately 8 days for all treatments in our experiment. This is considerably faster compared to laboratory results obtained by Svensson (2003) who found a 50% reduction of OA in *M. edulis* after 16 days. Lindahl & Hageltorn (1986) observed a 50% reduction after 1.5 months and Marcaillou-Le Baut *et al.* (1993) after 1 month of depuration in the laboratory. Similar depuration rates to those in this paper were observed by Blanco *et al.* (1999) ($t_{1/2}$=7-8 days), Fernández *et al.* (1998) ($t_{1/2}$=11-12 days) and Marcaillou-Le Baut *et al.* (1993) ($t_{1/2}$=12 days) for *M. galloprovincialis*. Croci *et al.* (1994) observed very high rates of depuration of DST in mussels (*M. galloprovincialis*) kept in ozonized water with a reduction of 50% after 3 days in two samples out of three.

High r^2 values (0.82-0.99) suggested that the depuration kinetics of OA for all treatments was accurately explained using a simple one-compartment model where depuration rate is constant over time (Spacie & Hamelink, 1985; Silvert & Cembella, 1995). This was also observed by Croci *et al.* (1994) and Blanco *et al.* (1995). A two-step depuration curve of DST, corresponding to a two-compartment model with an initial high depuration rate during the first days of depuration followed by a lower rate, has previously been observed by Bauder *et al.* (2001) in the digestive gland of scallops, *Agropecten irradians*, and in mussels *M. galloprovincialis* (Fernández *et al.*, 1998; Marcaillou-Le Baut *et al.*, 1993) and *M. edulis* (Svensson, 2003). Blanco *et al.* (1999) investigated the effects of environmental conditions on depuration rates of DST in *M. galloprovincialis* in the field and used both a one- and two-compartment model approach. They concluded that the most accurate model

describing depuration kinetics was dependent on wether the effects of environmental variables was included or not.

To summarize, different observations on both rates and depuration kinetics of DST in mussels indicate that further research into the physiological and biochemical processes, regulating accumulation and depuration of these compounds in mussels, is needed. Important is to identify the molecular binding site or storage location of the DST in mussel tissue. The turnover rate of these cellular or intracellular components may then be involved in regulating depuration rate of DST. The results in our experiment indicated that OA is not bound to lipid storage products in the digestive gland. An interesting theory was recently discussed by Córdova *et al.* (2001) who suggested that the intracellular protein phosphatases (PP) are the main target for OA in mussels and that some species are able to compensate for the resulting loss in PP activity by producing higher amounts of other PP isoforms, not sensitive to the binding of DST. If so, the turnover rate of PP could be involved in regulating the release of DST.

Finally, the occurrence and magnitude of biotransformation of OA, DTX-1 or DTX-2 to their respective acylated forms (DTX-3) as a route for elimination of DST in mussels should be explored. The toxicity of DTX-3 is considerably lower than the non-acylated forms due to a reduced binding-affinity for the PP (Yasumoto *et al.*, 1989; Yanagi *et al.*, 1989; Takai *et al.*, 1992), which means that acylation represents a detoxification pathway for bivalves. *Mytilus* sp. generally contains only low amounts of DTX-3 of the total DST present in tissue (Fernández *et al.*, 1996; Marr *et al.*, 1992; Fernández *et al.*, 1998; Suzuki & Mitsuya, 2001; Vale & Sampayo, 2002). So far, there is only one study which compares the detoxification kinetics between OA/DTX-2 and their respective acyl-derivatives in mussels, *M. galloprovincialis* (Fernández *et al.*, 1998). They found different depuration kinetics and considerably slower depuration rates for the acyl-derivatives compared to OA or DTX-2 which suggested that (1) rate of acylation is not constant (2) the mechanism and/or rate of tissue elimination differs between acyl-derivatives and parent toxins. In Swedish blue mussels, DTX-3 is currently not included in monitoring for DST and not much is known about the occurrence of DTX-3. A preliminary analysis of DTX-3 in subsamples from this current experiment showed that the proportion of acylated OA ranged between 25 to 74% (data not shown). Hence, significant amounts of DTX-3 may be present in Swedish mussels. Because acylated OA is assumed to rapidly hydrolyze to OA in the human stomach (T. Aune, pers. comm.), large amounts of acyl-OA imply an additional risk for consumers of mussels and need to be accounted for in monitoring of DST.

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References

Bauder, A. G., Cembella, A. D., Bricelj, V. M., Quilliam, M. A., 2001. Uptake and fate of diarrhetic shellfish poisoning toxins from the dinoflagellate *Prorocentrum lima* in the bay scallop *Agropecten irradians*. Mar. Ecol. Prog. Ser. 213, 39-52.

Bayne, B. L., Thompson, R. J., Widdows, J., 1973. Some effects of temperature and food on the rate of oxygen consumption by Mytilus edulis L. In: Wieser, W., (Ed.), Effects of temperature on ectothermic organisms. Springer-Verlag, Berlin, pp. 181-193.

Bayne, B. L. 1985. Responses to environmental stress: tolerance, resistance

and adaptation. Proc. 18th Eur. Mar. Biol. Symp. 331-349.

Blanco, J., Fernández, M., Marino, J., Reguera, B., Míguez, A., Maneiro, J., Cacho, E., Martínez, A., 1995. From *Dinophysis* spp. toxicity to DSP outbreaks: a preliminary model of toxin accumulation in mussels. In: Lassus, P., Arzul G., Erard E., Gentien P., Marcaillou C., (Ed.), Harmful marine algal blooms. Lavoisier, Intercept Ldt, Paris, pp. 777-782.

Blanco, J., Fernández, M. L., Míguez, A., Morono, A., 1999. Okadaic acid depuration in the mussel Mytilus galloprovincialis: one- and two-compartment models and the effect of environmental conditions. Mar. Ecol. Prog. Ser. 176, 153-163

Carmody, E. P., James, K. J., Kelly, S. S., 1996. Dinophysistoxin-2: The predominant diarrhoetic shellfish toxin in Ireland. . Toxicon 34 (3), 351-359.

Córdova, J. L., Bustamante, J., Cárdenas, L., 2001. Specific inhibition of endogenous shellfish protein phosphatase that could be used as a direct reporter of diarrhetic shellfish poison. J. Shellfish Res. 20 (1), 63-67.

Croci, L., Toti, L., De Medici, D., Cozzi, L., 1994. Diarrhetic shellfish poisoning in mussels: comparison of methods of detection and determination of the effectiveness of depuration. J. Int. Food Microbiol. 24, 337-342.

De Moreno, J. E. A., Pollero, R. J., Moreno, V. J., Brenner, R. R., 1980. Lipids and fatty acids of the mussel (*Mytilus platensis* dÓrbigny) from south atlantic waters. J. Exp. Mar. Biol. Ecol. 48, 263-276.

De Zwaan, A., Mathieu, M., 1992. Cellular energy metabolism in the Mytilidae: an overview. In: Gosling, E., (Ed.), The mussel Mytilus: ecology, physiology, genetics and culture. Elsevier, Amsterdam, pp. 223-307.

Farrington, J. W., 1989. Bioaccumulation of hydrophobic organic pollutant compounds. In: Levin, S. A., Harwell J. R., Kelly J. R., Kimball K. D., (Ed.), Ecotoxicology: Problems and Approaches. Springer-Verlag, New York, pp. 279-313.

Fernández, M. L., Míguez, A., Cacho, E., Martínez, A., 1996. Detection of okadaic acid esters in the hexane extracts of Spanish mussels. Toxicon 34 (3), 381-387.

Fernández, M. L., Míguez, A., Morono, A., Cacho, E., Martínez, A., Blanco, J., 1998. Detoxification of low polarity toxins (DTX-3) from mussels *Mytilus galloprovincialis* in Spain. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 449-452.

Fujiki, H., Suganuma, M., 1993. Tumor promotion by inhibitors of protein phosphatase 1 and 2A: the okadaic acid class of compounds. Adv. Canc. Res. 61, 143-194.

Gardner, W. S., Frez, W. A., Cichocki, E. A., 1985. Micromethod for lipids in aquatic invertebrates. Limnol. Oceanogr. 30 (5), 1099-1105.

Haamer, J., Andersson, P-O., Lange, S., Li, X.P., Edebo, L., 1990. Effects of transplantation and reimmersion of mussels Mytilus edulis L. on their contents of okadaic acid. J. Shellfish Res. 9 (1), 109-112

Hawkins, A. J. S., Bayne, B. L., 1984. Seasonal variation in the balance between physiological mechanisms of feeding and digestion in *Mytilus edulis* (Bivalvia: Mollusca). Mar. Biol. 82, 233-240.

Hawkins, A. J. S. 1985. Relationships between the synthesis and breakdown of protein, dietary absorption and turnovers of nitrogen and carbon in the blue mussel, *Mytilus edulis* L. Oecologia 66, 42-49.

Hawkins, A. J. S., Wilson, I. A., Bayne, B. L., 1987. Thermal responses reflect protein

turnover in *Mytilus edulis* L. Funct. Ecol. 1, 339-351.

Hawkins, A. J. S., Bayne, B. L., 1992. Physiological interrelations, and the regulation of production. In: Gosling, E., (Ed.), The mussel Mytilus: ecology, physiology, genetics and culture. Elsevier, Amsterdam, pp. 171-222.

Kollberg, S. 1999. Beskrivning av den svenska musselnäringen (In Swedish). Vattenbrukarnas Riksförbund.. 68 pp.

Kumagai, M., Yanagi,T., Murata,M., Yasumoto,T., Kat,M., Lassus,P., Rodriguez-Vazquez,J.A., 1986. Okadaic acid as the causative toxin of diarrhetic shellfish poisoning in Europe. Agric.Biol.Chem. 50 (11), 2853-2857.

Lee, J. S., Yanagi, T., Kenma, R., Yasumoto, T., 1987. Fluorometric determination of diarrhetic shellfish toxins by high pressure liquid chromatography. Agr. Biol. Chem. 51, 877-881.

Lindahl, O., Hageltorn, M. 1986. Detoxification experiments of DSP in blue mussels. 15th Nordic Veterinary Congress. pp. 463-466.

Lindegarth, M. 1997. Sammanställning och tolkning av de kontinuerliga mätningarna av okadasyrahalter längs den svenska västkusten (In Swedish). Länsstyrelsen Västra Götaland. 17 pp.

Livingstone, D. R., Pipe, R. K., 1992. Mussels and environmental contaminants: molecular and cellular aspects. In: Gosling, E., (Ed.), The mussel Mytilus: ecology, physiology, genetics and culture. Elsevier Science Publishers, Amsterdam, pp. 425-464.

Marcaillou-Le Baut, C., Bardin, B., Bardouil, M., Bohec, M., Le Dean, L., Masselin, P., Truquet, P., 1993. DSP depuration rates of mussels reared in a laboratory and an aquaculture pond. In: Smayda, T. J., Shimizu Y., (Ed.), Toxic phytoplankton blooms in the sea. Elsevier Science Publisher, pp. 531-535.

Marr, J. C., Hu, T., Pleasance, S., Quilliam, M. A., Wright, J. L. C., 1992. Detection of new 7-*O*-acyl derivatives of diarrhetic shellfish poisoning toxins by liquid chromatography-mass spectrometry. Toxicon 30 (12), 1621-1630.

Morono, A., Fernández, L. M., Franco, J. M., Martínez, A., Reyero, I. M., Míguez, A., Cacho, E., Blanco, J., 1998. PSP and DSP kinetics in mussel, *Mytilus galloprovincialis*: effect of environmental parameters and body weight. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 445-448.

Murakami, Y., Oshima, Y., Yasumoto, T., 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. Bull. Jpn. Soc. Sci. Fish. 48, 69-72.

Murata, M., Shimatani, M., Sugitani, H., Oshima, Y., Yasumoto, T., 1982. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. Bull. Japan Soc. Sci. Fish. 48, 549-552.

Phillips, D. J. H., 1993. Bioaccumulation. In: Calow, P., (Ed.), Handbook of ecotoxicology. Blackwell Scientific Publications, pp. 378-398.

Poletti, R., Viviani, R., Casadei, C., Lucentini, L., Funari, E., Draisci, R., 1996. Decontamination dynamics of mussels naturally contaminated with diarrhetic toxins relocated to a basin of the Adriatic sea. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 429-432

Sampayo, M. A., Alvito, P., Franca, S., Sousa, I., 1990. *Dinophysis* spp. toxicity and relation to accompanying species. In: Granéli, E., Sundström B., Edler L., Anderson D. M. M., (Ed.), Toxic marine phytoplankton. Elsevier, New York, pp. 215-220.

Shumway, S. E., van Egmond, H. P., Hurst, J. W., Bean, L. L., 1995. Management of shellfish resources. In: Hallegraeff, G. M., Anderson D. M., Cembella A., (Ed.), Manual on harmful marine microalgae. UNESCO, Paris, pp. 433-462.

Silvert, W. L., Cembella, A. D., 1995. Dynamic modelling of phycotoxin kinetics in the blue mussel, *Mytilus edulis*, with implications for other marine invertebrates. Can. J. Fish. Aquat. Sci. 52, 521-531.

Spacie, A., Hamelink, J. L., 1985. Bioaccumulation. In: (Ed.), Fundaments of aquatic toxicology. Hemisphere Publ. Corp., New York, pp. 495-525.

Suzuki, T., Mitsuya, T., 2001. Comparison of dinophysistoxin-1 and esterified dinophysistoxin-1 (dinophysistoxin-3) content in the scallop *Pactinopecten yessoensis* and the mussel *Mytilus galloprovincialis*. Toxicon 39, 905-908.

Svensson, S., 2003. Depuration of okadaic acid (diarrhetic shellfish toxin) in mussels, *Mytilus edulis* (Linnaeus), feeding on different quantities of non-toxic algae. Aquaculture 218, 277-291

Takai, A., Murata, M., Torigoe, K., Isobe, M., Mieskes, G., Yasumoto, T., 1992. Inhibitory effects of okadaic acid derivatives on protein phosphatases. A study on structure-affinity relationship. Biochem. J. 284, 539-544.

Thompson, R. J., Ratcliffe, N. A., Bayne, B. L., 1974. Effects of starvation on structure and function in the digestive gland of the mussel (*Mytilus edulis* L.). J. mar. biol. Ass. U.K. 54, 699-712.

Underwood, A. J., 1997. Experiments in ecology; their logical design and interpretation using analysis of variance, ed. Cambridge University Press, Cambridge, UK.

Vale, P., Sampayo, M. A., Quilliam, M. A., 1998. DSP complex toxin profiles relation with Dinophysis spp. occurrence and domoic acid confirmation by LC-MS in Portugese bivalves. In: Reguera, B., Blanco J., Fernandez M. L., Wyatt T., (Ed.), Harmful algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 503-506.

Vale, P., Sampayo, M. A. M., 2002. Esterification of DSP toxins by Portugese bivalves from the Northwest coast determined by LC-MS-a widespread phenomenon. Toxicon 40, 33-42.

Vieites, J. M., Leira Sanmartin, F., 2000. Incidence of marine toxins on industrial activity. In: Botana, L. M., (Ed.), Seafood and freshwater toxins. Pharmacology, physiology and detection. Marcel Dekker, New York, pp. 741-760.

Vieytes, M. R., Fontal, O. I., Leira, F., Baptista de Sousa, J. M. V., Botana, L. M., 1997. A fluorescent microplate assay for diarrheic shellfish toxins. Anal. Biochem. 248, 258-264.

Widdows, 1973. Effects of temperature and food on the heart beat, ventilation rate and oxygen uptake of *Mytilus edulis*. Mar. Biol. 20, 269-276.

Widdows, J., Bayne, B. L., 1971. Temperature acclimation of *Mytilus edulis* with reference to its energy budget. J. mar. biol. Ass. U.K. 51, 827-843.

Wieser, W., 1973. Temperature relations of ectotherms: a speculative view. In: Wieser, W., (Ed.), Effects of temperature on ectothermic organisms. Springer-Verlag, Berlin, pp. 1-24.

Yanagi, T., Murata, M., Torigoe, K., Yasumoto, T., 1989. Biological activities of semisynthetic analogs of Dinophysistoxin-3, the major diarrhetic shellfish toxin. Agric. Biol. Chem. 53 (2), 525-529.

Yasumoto, T., Oshima, Y., Yamaguchi, M., 1978. Occurence of a new type of shellfish poisoning in the Tohoku district. Bulletin of the Japanese Society of Scientific Fisheries 44 (11), 1249-1255.

Yasumoto, T., Oshima, Y., Sugawara, W., Fukuyo, Y., Oguri, H., Igarashi, T., Fujita,

N., 1980. Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. Bull. Jpn. Soc. Sci. Fish. 46, 1405-1411.

Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G. K., Clardy, J., 1985. Diarrhetic shellfish toxins. Tetrahedron 41, 1019-1025.

Yasumoto, T., Murata, M., Lee, J. S., Torigoe, K., 1989. Polyether toxins produced by dinoflagellates. In: Natori, S., Hashimoto K., Ueno Y., (Ed.), Mycotoxins and phycotoxins ´88. Elsevier, Amsterdam, pp. 375-382.

Zaba, B. N., Davies, J. I., 1984. Glycogen metabolism and glucose utilisation in the mantle tissue of *Mytilus edulis*. Mol. Physiol. 5, 261-282.

V

LARGE-SCALE DEPURATION OF OKADAIC ACID (DIARRHETIC SHELLFISH TOXIN) IN FARMED MUSSELS, *MYTILUS EDULIS*, BY RELOCATION TO A FJORD AREA ON THE SWEDISH WEST COAST

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*Abstract***:** Large-scale depuration of mussels (*Mytilus edulis*) containing the diarrhetic shellfish toxin (DST) okadaic acid (OA) was conducted in a fjord system on the Swedish west coast, where native mussels rarely contain high concentrations of DST. Toxic mussels (600 µg OA*kg^{-1}) were relocated from a long-line farm in the outer archipelago to the fjord. They were kept in large tanks, supplied with a continuous flow of natural seawater, and depurated for five weeks. Weekly sampling for OA and DTX-1 in mussels together with plankton toxicity and identification was performed both in the fjord and the farm area. A fast, exponential reduction of OA was observed during the first three weeks in mussels relocated to the fjord. Depuration half-life was calculated to seven days. After twelve days, OA levels in the depurated mussels were below the regulation limit for marketing (160 µg OA*kg^{-1}). Concentrations of OA in farm mussels remained more or less constant throughout the period, indicating a continuous ingestion of toxic algae in this area. Although OA was efficiently eliminated in the fjord, mussels accumulated DTX-1 during the experiment. A peak in DTX-1 was observed after five weeks when mussels were again unsuitable for consumption. Analysis of plankton data indicated that plankton toxicity was poorly correlated to cellular abundance of *Dinophysis* sp. suggesting that toxin content per cell varied during the experiment. However, we could confirm that *Dinophysis acuta* was the causative species for DTX-1 production in the fjord. DTX-1 was never detected in the farm mussels even though *D. acuta* was present in substantial numbers. This geographical difference in toxin profiles suggested that (1): two genetically distinct populations exist along the coast, or (2): differences in environmental conditions between the coastal areas and the fjord system affect the production of DST components in *D. acuta*. It was concluded that depuration of OA can be effective as long as the water column is free of toxin-producing algae.

Keywords: diarrhetic shellfish toxins, Dinophysis, depuration, large-scale experiment, mussel, okadaic acid, plankton, relocation

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1. Introduction

Harmful and toxic algal blooms (HAB) are a global problem with considerable negative effects on fisheries and aquaculture of bivalve molluscs such as mussels (Hallegraeff, 1995; Shumway *et al.*, 1995; Vieites & Leira Sanmartin, 2000). Today, most countries where shellfish resources are used as human food have ongoing regular monitoring programs to detect HAB toxins in mussels which prevents unsafe mussels to reach the market in most cases (Shumway *et al.*, 1995; Andersen, 1996). Although regular analysis of shellfish generally protects consumers from the effects of HAB, the people involved in fisheries and aquaculture activities may suffer great economical losses due to extensive periods of toxic mussels. For example, the outbreak of diarrhetic shellfish poisoning (DSP) along the west coast of Sweden in 1984 had devastating effects on the expanding mussel (*Mytilus edulis*) industry which almost disappeared for several years (Krogh *et al.*, 1985; Underdal *et al.*, 1985). Monitoring for DSP toxins (DST) since 1985 has revealed that okadaic acid (OA) is the dominant DST in Swedish mussels which correlate to the occurrence of *Dinophysis acuta* and *D. acuminata* (Kumagai, 1986). Large systematic seasonal and geographical variations in OA concentration occur (Edebo, 1988; Haamer, 1990b; Lindegarth, 1997). In general, levels of OA are high during late autumn and winter and mussels in fjord areas, which are somewhat sheltered from the outer sea, are less affected by high concentrations of DST. Although a re-establishment of mussel farming activities is occurring today, the presence of DST in mussels is still a major problem for this industry in Sweden (Kollberg, 1999).

To reduce the effects of DST, or other HAB toxins, on the mussel farming industry, different approaches to the problem may be employed. For example, various strategies to prevent the toxins from accumulating in the mussels can be used. These may include increased efforts to find locations for mussel farms where toxic dinoflagellates are less prevalent although there is no 100% guarantee that a certain area will be unaffected by future HAB. Also, use of reliable early warning systems for toxic events gives mussel farmers opportunities to take measures before the mussels become intoxicated. A different approach is to depurate toxic mussels, which would allow a more continuous supply of mussels. For this purpose, access to seawater free of toxin-producing dinoflagellates is a prerequisite. In general terms, mussels depurate from DST when ingestion of toxic dinoflagellates is reduced or stopped but large, usually seasonal, variability in rates of depuration is observed in the field. To develop practical and cost-effective methods for depuration, extensive knowledge about variations in rates of depuration during different seasons and environmental conditions are needed.

The aim of this paper was to test possibilities for and efficiency of large-scale depuration for mussels containing okadaic acid. Previous studies and monitoring of DST in mussels growing in fjords north of the island of Orust on the Swedish west coast has revealed consistently lower, or non-detectable, levels of DST compared to mussels from the outer archipelago. Thus, this was considered a suitable area for depuration (Haamer, 1995; Lindahl & Andersson, 1996; Lindegarth, 1997). Toxic mussels from a long-line farm were relocated to the fjord and depurated for five weeks in tanks supplied with natural seawater. Weekly sampling for DST in both mussels and plankton extracts together with plankton counts and identification was performed at both sites. The influence of non-toxic food resources and proportion of *Dinophysis* of the total plankton

community for depuration efficiency was evaluated.

2. Methods

2.1. Animals and experimental set up

Mussels (*M. edulis*, 70-80 mm shell length, 2 years old) were harvested on 30th of August, 2000, from a commercial long-line mussel farm situated in the Hamburgsund area in the outer archipelago (Fig. 1). The mussels contained okadaic acid (OA) as the only DST (600 µg OA*kg^{-1} mussel meat). Mussels were removed from the growth bands with care not to damage the shells. They were immediately transported by truck to the experimental site, located in Kalvöfjorden (Fig. 1). This fjord system north of the island of Orust is characterized by a restricted exchange of seawater from the outside area because of narrow straits and shallow sills (Haamer, 1995). The experiment was conducted in a shallow cove on a jetty at Såtas wharf. Two large tanks, each with a volume of 1 m^3, was installed next to the water. These tanks were originally designed for the purpose of removing sand from cockles (Franken BV, Holland). Approximately 400-500 kg of mussels were put in each tank. Seawater was pumped from 1-2 m depth through pipes into the tanks using a high capacity submerged pump with a flow velocity of approximately 100 l*min^{-1} (bottom-up flow direction). No increased mortality occurred in the tanks during the experiment. Water temperature was measured once a week at both sites (Table 4). The experiment was conducted for five weeks with a weekly sampling frequency both at the farm site and the experimental site. At each occasion, analysis of DST in mussels and plankton extracts together with plankton counts and identification was carried out. Also, changes in the condition of the mussels were estimated by measuring the meat yield and dry weights.

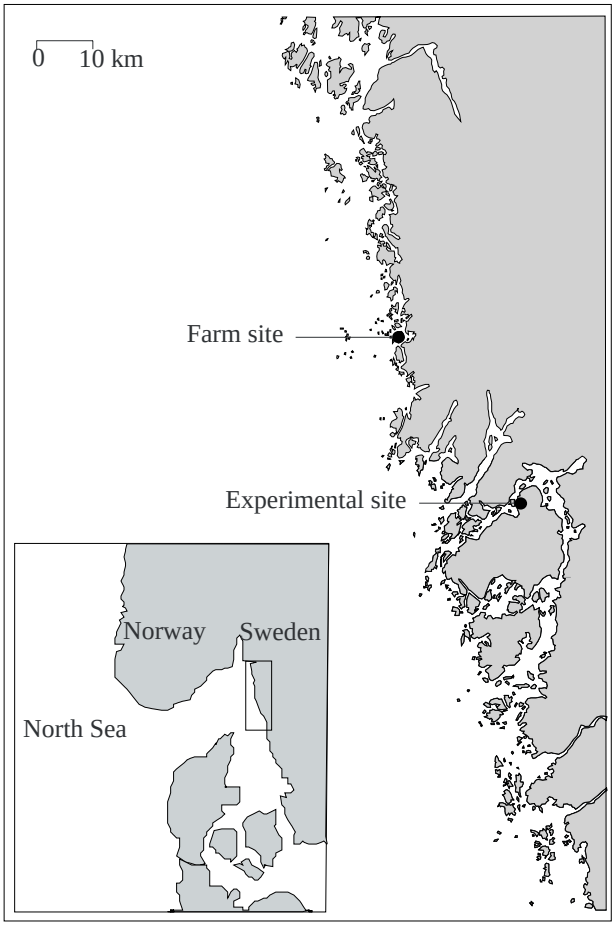

Fig. 1. Location of Grisholmen long-line mussel farm in the Hamburgsund region (farm site) and the depuration area in Kalvöfjorden (experimental site) north of the island of Orust.

2.2. Analysis of DST in mussels

Replicate samples (n=2), each consisting of twelve pooled mussels, were randomly taken from each of the tanks at the experimental site or from three different depths within the farm. The digestive glands were removed, weighed and the proportion digestive gland of total flesh weight was determined. Analysis of OA and DTX-1 was done using HPLC according to the method of Lee *et al.* (1987) with minor modifications (Edebo, 1988). 1-pyrenyl-diazomethan (PDAM) was used as a derivatization reagent instead of ADAM. The toxin concentration was expressed as µg OA or DTX-1*kg^{-1} mussel meat. Current upper limit for marketing of mussels within European Community is 160 µg total DST*kg^{-1} mussel meat.

2.3. Phytoplankton sampling

Water samples for plankton species identification, cell counts and plankton toxicity was collected at the same day as the mussels were sampled. At the farm site, integrated water samples from 1 to 8 meters depth were collected. At the experimental site, seawater was collected from the pump system (1-2 m depths) before passing over the mussel tanks and a defined volume between 30-80 litres of water was filtered through a 10 µm mesh. From this filtrate, 100 ml were preserved using Lugholes iodine (1-2% final concentration) in brown glass bottles. The samples were stored in room temperature and analysed within 2 months. For microscopic studies, 1 ml of each sample were filtrated through a Whatman GF/F filter. A drop of oil were added to the filter before the coverslip were added, this prevented the sample to get dry during the examination. All cells present on the filter were counted. A standard laboratory microscope (Leitz) was used at 100x and 200x magnification.

Biovolumes were calculated as geometric bodies according to Edler (1977) and Hansen

Table 1. Plankton species (>10 µm) identified during the experiment together with data on biovolumes. Species were divided into five groups and the contribution of each group to the total biomass was calculated.

Species	Volume (μm^3)	Group	Comment
Ceratium furca	60000	Dino.	Hansen (1992)
Ceratium tripos	130000	Dino.	Hansen (1992)
Ceratium lineatum	28000	Dino.	Hansen (1992)
Ceratium fusus	23000	Dino.	Hansen (1992)
Prorocentrum micans	9300	Dino.	Hansen (1992)
Dinophysis acuta	40000	Dino.	According to Edler (1977)
Dinophysis acuminata	12600	Dino.	w= 30µm, l=40µm, t=20µm, V=´/6*w*l*t
Dinoshysis norvegica	31400	Dino.	w= 40µm, l=50µm, t=30µm, V=´/6*w*l*t
Dinophysis rotundata	33500	Dino.	w=40µm, V=´/6*w^3
Dinophysis dens	12600	Dino.	According to Edler (1977)
Polykrikos schwartzii	100000	Dino.	w=50µm, l=100µm, t=20, V=w*l*t
Protoperidinium sp.	175000	Dino.	Calculated as *P. depressum*, Edler (1977)
Gonyaulax grindley	16400	Dino.	According to Edler (1977)
Leptocylindrus sp.	53000	Diatom	w=15µm, l=300µm
Chaetoceros sp.	4855	Diatom	Calculated as the mean value of Edler (1977) Chaetoceros species when <10µ were excluded.
Dictyocha speculum	4000	Other	According to Edler (1977)
Skeletonema sp.	850	Diatom	According to Edler (1977)
Proboscia sp.	8800	Diatom	According to Edler (1977)
Tintinnida ciliater	50000	Other	Approximated from Edler (1977)
Cylindrotheca sp.	200	Diatom	Approximated from Edler (1977)
Thalassiosira sp.	19000	Diatom	Approximated from Edler (1977)
Pseudonitzschia	500	Diatom	Approximated from Edler (1977)
Nitzschia sp.	500	Diatom	Approximated from Edler (1977)
Spirulina	500	Other	Approximated from Edler (1977)
Fragilaria sp.	1000	Diatom	Approximated from Edler (1977)
Amphora sp.	3000	Diatom	Approximated from Edler (1977)
Biddulphia	3000	Diatom	Approximated from Edler (1977)
Parvilucifera sporangia	1000	Other	Approximated from Edler (1977)
Noctiluca	900000	Dino.	Approximated from Edler (1977)
Achnantes sp.	400	Diatom	Approximated from Edler (1977)

(1992) and used as a measure of biomass (Hansen, 1992). A summary of values used is presented in Table 1. Some volumes were only approximately calculated due to their minute contribution to the total biomass and hence of minor importance. The plankton were divided into six groups; *Dinophysis acuta, Dinophysis acuminata, Dinophysis norvegica,* Dinoflagellates, Diatoms and Others. The contribution of each group to the total biomass was analysed. The three *Dinophysis* species were not included in the group Dinoflagellates. The species included in each group are listed in Table 1. All plankton samples were analysed in duplicates except for week 4 at the control site where one sample was lost. Thus, week 4 was excluded from the statistical analysis of the various plankton measures.

2.4. Toxicity in plankton extracts

For analysis of toxicity in the plankton community, samples were collected in the same manner as described for cell count and identification. The plankton concentrates were transferred into 50 ml Falcon test tubes and centrifuged at 3000 rpm for 5 minutes. The resulting algal pellets were stores at -80°C until analysis of DST toxicity was done using the protein phosphatase inhibition assay (PPIA) developed by Vieytes *et al*. (1997) and modified for algal samples by (Godhe *et al.*, 2002). The concentration of DST in the plankton extracts were expressed as ng OA equivalents*l^{-1} seawater (ng OA eq.*l^{-1}).

2.5. Analysis of meat yield and dry weights

The meat yield and dry weights were estimated and used as a measure of quality. 12 individual mussels were randomly sampled from each tank or from different depths within the mussel farm. To analyse the meat yield, the mussels were boiled for five minutes and the weight of the separated mussel meat and the shells was determined. Meat yield was expressed as % meat of total weight. Dry flesh weights were determined by removing the shells and drying the mussel meat in 80°C for three days and then weighing the remaining dried flesh (g).

2.6. Statistical analysis

Two-factor (fixed effects) analysis of variance (ANOVA) was used to test differences between locations and weeks of depuration for various variables: concentration of OA in mussels, plankton toxicity, *Dinophysis* spp. abundance and total biovolume of plankton. The difference in OA concentration in mussels between the tanks at the experimental site was tested separately. No difference was detected (tank*week, $p=0.86$), thus the factor tank was not included in the full analysis. Data was checked for homogeneity of variances using Cochran´s test and for significant effects, Student-Neuman-Keuls (SNK) *a posteriori* test for differences among means was used. For analysis of abundance of *Dinophysis* spp. and total biovolume of plankton, week four was excluded from the statistical analysis due to a lost replicate from the control site.

3. Results

3.1. Depuration efficiency of OA

At the start of the experiment, mussels contained 600 µg OA*kg^{-1} mussel meat which was approximately four times above the limit for marketing (Fig. 2). Already after one week of depuration, OA concentration was significantly lower in the relocated mussels (261±112 SE µg OA*kg^{-1}) compared to mussels at the farm site (587±129 µg OA*kg^{-1}) (Table 2, $p<0.05$ for L*W). Whereas OA was further reduced after two and three weeks in fjord mussels, concentration of OA remained high in farm mussels until the last sampling occasion,

when a significant reduction was observed. During the first three weeks of the experiment, a constant depuration rate (exponential decrease) of OA was observed in fjord mussels. The data was fitted to the exponential loss equation, $T_t=T_0*e^{-\lambda t}$ (T_0=toxin concentration at the start of depuration (µg OA*kg^{-1}), λ=exponential decay coefficient (day^{-1}) and t=time (days)), and the high score for the correlation coefficient (r^2=0.99) indicated that this model accurately described the depuration kinetics during this period. From this relationship, the depuration half-life of OA was estimated to seven days. Considering that the start value was 600 µg OA, it would have been possible to market the depurated mussels after twelve days since the concentration of OA was calculated to be below the regulation limit by then (160 µg OA*kg^{-1} mussel meat).

3.2. Accumulation of DTX-1 in relocated mussels

At the start of the experiment, DTX-1 was not present in the mussels. However, after one week in the fjord site, low amounts of DTX-1 were detected in the relocated mussels (10±7 µg DTX-1*kg^{-1}, Fig. 2). The uptake of DTX-1 slowly continued and between week four and five, a rapid increase from 84±13 to 356±33 µg DTX-1*kg^{-1} occurred. The accumulation of DTX-1 resulted in mussels being unsuitable for consumption after five weeks at the experimental site. DTX-1 was never detected in the mussels at the farm site.

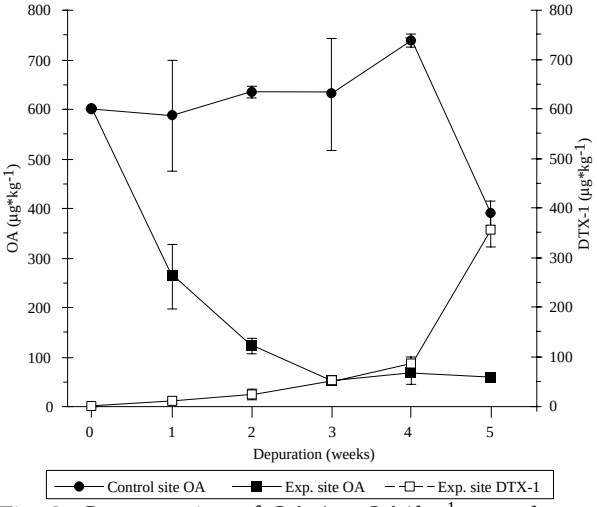

Fig. 2. Concentration of OA (µg OA*kg^{-1} mussel meat) in mussels at the control and experimental site, primary y-axis. Concentration of DTX-1 (µg DTX-1*kg^{-1} mussel meat) in mussels at the experimental site, secondary y-axis.

3.3. Occurrence of Dinophysis spp. and toxicity in plankton extracts

Dinophysis acuta, *D. acuminata* and *D. norvegica* were present in various concentrations at both sites during the experimental period (Fig. 3A, B). On average, *D. acuta* was the most abundant species at both locations during the experiment. Similar concentrations of *D. acuta* were detected at both locations during the experiment (Table 2, p>0.05 for Location). However, the mean abundance of *D. acuta* was generally higher in the fjord compared to the farm site with the exception of week three, when high concentrations (1 650±602 SE cells*l^{-1}) were detected in the farm area. In the fjord, >1000 *D. acuta**l^{-1}

Table 2. ANOVA on the effects of location (control vs experimental site) and depuration time (weeks) on mussel toxicity (OA, µg*kg^{-1} mussel meat) and plankton toxicity (OA equivalents, ng*l^{-1} sea water). Note: n=2 at the control site and n=4 at the experimental site for all comparisons in the analysis of mussel toxicity. In the analysis of plankton toxicity, n=2 for all comparisons.

		Mussel toxicity			Plankton toxicity		
Source	df	MS	F	p	MS	F	p
Location (L)	1	$1.56*10^6$	277.0	0.0001	0.40	1.45	0.26
Week (W)	4	$33.0*10^3$	5.87	0.0027	1.34	4.87	**0.02**
L* W	4	$31.0*10^3$	5.52	**0.0037**	0.31	1.14	0.39
Residual	20	$5.61*10^3$			0.27		

were observed at three sampling occasions. Concentration of *D. acuta* in this area was higher at all occasions compared to *D. acuminata*, which never exceed 200 cells*l^{-1}. At week four, no *D. acuminata* were present in the plankton samples. Significantly higher concentrations of *D. acuminata* (1 319±200 cells*l^{-1}) were observed in the farm compared to the fjord at week two (Table 2, $p<0.05$ for L*W). Also, significantly higher numbers of *D. norvegica* (3 622±1 621 cells*l^{-1}) was found at week three at the farm site (Table 2, $p<0.05$ for L*W). High concentrations of *D. norvegica* was present at the fjord site at week four (3 234±1 136 cells*l^{-1}), however, this occasion was not included in the statistical test.

Toxicity in plankton extracts varied between 0-1.32 ng OA eq.*l^{-1} seawater on average during the experiment (Fig. 3A, B). In the farm site, toxicity was detected at all occasions and at week four, a peak was observed (1.13±0.89 ng*l^{-1}). In the fjord, plankton toxicity was below the detection limit during the first two weeks. Low toxicity was detected in the extracts at week three but during the last two experimental weeks, plankton toxicity increased considerably, peaking at week four with a toxicity of 1.32±0.71 ng*l^{-1}. A statistically significant difference in plankton toxicity was detected at week four, when toxicity was significantly higher in both locations compared to all other weeks (Table 2, $p<0.05$ for Week). *D. norvegica* is generally not associated with toxic mussels along the Swedish west coast, thus we focused on *D. acuta* and *D. acuminata* to study the relationship between *Dinophysis* and toxicity in the plankton extracts. In the farm, there was no apparent positive correlation between either the concentration of *D. acuminata* or *D. acuta* and plankton toxicity. The highest values of plankton toxicity was observed at week one and four when cell numbers were generally low. The opposite was observed at week three when very low toxicity was detected in an extract containing high concentrations of *D. acuta*. This pattern indicated that toxin production per cell varied among sampling occasions. In the fjord, a positive correlation between concentration of *D. acuta* and toxicity in the plankton extracts was observed at week four and five. Since a rapid accumulation in DTX-1, but not in OA, occurred in the mussels between these occasions, it was concluded that *D. acuta* was the causative species for this, producing DTX-1 as a major toxic component in the fjord area. It should also be noted that the plankton extracts were non-toxic at week two in the fjord even though relatively high numbers of *D. acuta* (1 180±917 cells*l^{-1}) were present in the sample, suggesting temporal variability in toxin production for this species in the fjord. The *D. acuta* population in the farm did not appear to produce DTX-1 since this toxin was never detected in mussels from this site.

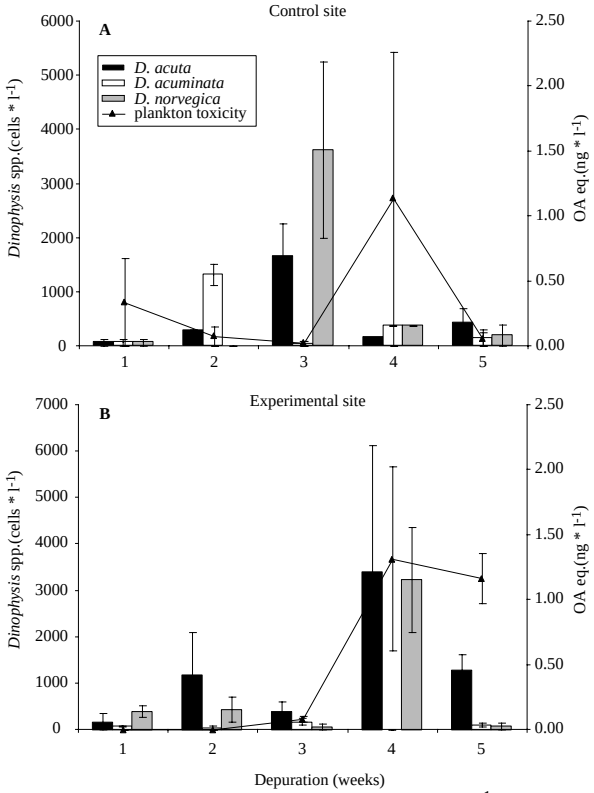

Fig. 3. Abundance of *Dinophysis* sp. (cells*l^{-1} seawater, primary y-axis) and plankton toxicity (ng OA equivalents*l^{-1} seawater, secondary y-axis) during the experiment at A. the control site and B. the experimental site.

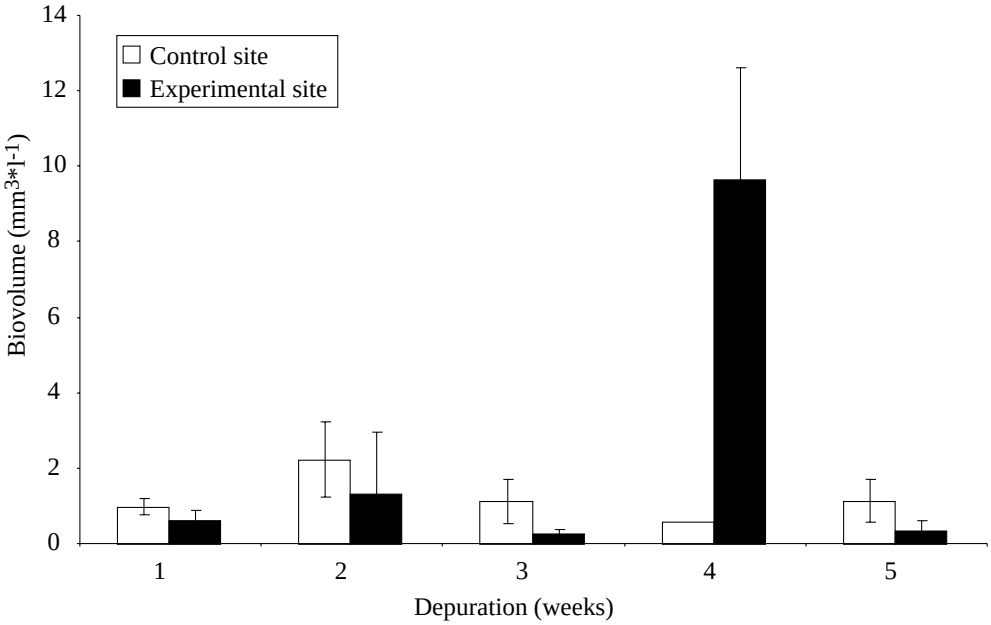

Fig. 4. Biovolume of the plankton community >10 µm (mm3*l^{-1} seawater) at both sites during the experimental period.

3.4. Plankton biomass and Dinophysis in the community

Using biovolumes as a measure of biomass, total plankton biomass (Fig. 4, Table 3) and proportional contribution of *Dinophysis* to the total community (data not shown) was estimated. Total biomass was found to be similar at both sites throughout the experiment (Table 3, p>0.05 for all factors) although there was an indication that the biomass was slightly higher at the control site (p=0.09 for Location). This is with the exception of week four, when biomass was substantially higher in the fjord (week four not included in the statistical analysis). The dinoflagellates were the main constituents of the plankton community during the whole period (79±26% S.D.) The percentage contribution of *D. acuta* ranged between 0.3-15.9% which was significantly higher than *D.*

Table 3. ANOVA on the effects of location (control vs experimental site) and depuration time (weeks) on the abundance of *Dinophysis acuta*, *D. acuminata* and *D. norvegica* (cells*l^{-1} sea water) and total biovolume of plankton > 20 µm (µm^3*l^{-1} sea water), n=2 for all comparisons. Note: week four not included in the analysis.

		D. acuta			*D. acuminata*		
Source	df	MS	F	p	MS	F	p
Location (L)	1	$92.3*10^3$	0.25	0.63	$0.35*10^6$	18.0	0.0028
Week (W)	3	$0.63*10^6$	1.72	0.24	$0.34*10^6$	17.6	0.0007
L* W	3	$1.00*10^6$	2.72	0.11	$0.43*10^6$	22.5	**0.0003**
Residual	8	$0.37*10^6$			$19.3*10^3$		

		D. norvegica			*Total biovolume*		
Source	df	MS	F	p	MS	F	F p
Location (L)	1	$2.12*10^6$	3.07	0.12	$2.08*10^{18}$	3.60	0.09
Week (W)	3	$2.71*10^6$	3.93	0.05	$1.01*10^{18}$	1.75	0.23
L* W	3	$3.63*10^6$	5.26	**0.03**	$0.69*10^{15}$	0.12	.95
Residual	8	$0.69*10^6$			$0.59*10^{18}$		

Table 4. Water temperature (°C), meat yield (% flesh of total weight) and soft tissue dry weights (g) in mussels at the farm site and in the experimental area during the experiment.

	Water temperature (°C)		Meat yield (%)		Dry weight (g)	
Depuration (weeks)	*Farm site*	*Exp. site*	*Farm site*	*Exp. site*	*Farm site*	*Exp. site*
1	15	16.5	35	37	2.80	2.10
2	13.5	14	37	31	2.51	2.07
3	13	14	38	31	2.29	1.94
4	13	12	44	28	3.04	2.19
5	11	11	42	26	3.35	2.23

acuminata (0-0.83%) at both locations throughout the experiment. This was with the exception of week two at the control site, when the proportion of *D. acuta* was lower than *D. acuminata*. The proportion of *D. acuta* was also significantly higher in the fjord area at week two and week five compared to the control site.

3.5. Mussel quality

Changes in meat yield and dry weights of mussels during the experiment are shown in table 4. The average meat yield was reduced from 37 to 31% in mussels at the experimental site after three weeks of depuration. Meat yield was further reduced during the last weeks of the experiment. Dry weights of the experimental mussels were lower at all sampling occasions comapared to the farmed mussels. This indicated that mussels were stressed and were losing body mass at the experimental site. However, a meat yield index of approximately 30% is considered to be acceptable for marketing of mussel (Z. Uggla, pers. comm.).

4. Discussion

Relocation of toxic mussels to non-toxic environments for depuration is a future management option for the shellfish industry. Crucial is to find areas where the toxin-producing *Dinophysis* spp. are less prone to occur. A general geographical pattern seems to exist where fjords, bays and estuaries with a restricted exchange of seawater are less affected by DST (Sedmak & Fanuko, 1991; Haamer, 1995; Poletti *et al.*, 1996). It has been suggested that blooms of *Dinophysis* originate in the sea and are transported to coastal areas by currents where they affect mussel farming activities (Edebo, 1991; Lassus *et al.*, 1993; Reguera *et al.*, 1995; Godhe *et al.*, 2002). However, in a deep fjord on the Norwegian west coast, the toxicity in mussels is generally larger inside the fjord than closer to the mouth which suggest that local blooms of *Dinophysis* may also occur (Aune *et al.*, 1996; Ramstad *et al.*, 2001).

A previous attempt to depurate mussels from OA by relocation from a toxic to a less toxic environment on the Swedish west coast was performed by Haamer (1990a). Fluctuations in OA concentrations were observed between days but a general reduction of 50% was achieved in the less toxic environment after 11 days of depuration.

In this experiment, a fast and efficient reduction of OA was obtained in mussels relocated to Kalvöfjorden for depuration. This factual depuration success was evaluated by comparing the toxicity of mussels at the farm site from where the experimental mussels originated. In the farm, levels of OA remained more or less constant in the mussels throughout the experiment, indicating a steady-state relationship between ingestion and depuration rates of OA. In the fjord, an exponential decrease of OA was observed during the first three weeks with a depuration half-life ($t_{1/2}$) of seven days. This implied that the mussels could have been marketed after

twelve days of depuration without any negative effects on the product quality. This is a relatively high rate of reduction compared to previous attempts to depurate *Mytilus* sp. containing DST. Considerable variability in depuration rates have been reported and observed half-lives for *M. edulis* have been 30-45 days (Lindahl & Hageltorn, 1986; Marcaillou-Le Baut *et al.*, 1993), 16 days (Svensson, 2002) and 12 days (Marcaillou-Le Baut *et al.*, 1993). Faster rates have been reported for *M. galloprovincialis* by (Blanco *et al.*, 1999) ($t_{1/2}$=7-8 days) and (Fernández *et al.*, 1998) ($t_{1/2}$=11-12 days). Very high rates of depuration of DST ($t_{1/2}$=3 days) were detected by Croci *et al.*, (1994) for *M. galloprovincialis*.

Differences in environmental conditions and whether depuration is performed in the field or in the laboratory may perhaps explain some of the variability in depuration rates of DST in mussels. However, depuration may be equally effective in the laboratory as in the field. Svensson & Förlin (In prep.) performed a laboratory experiment in parallel to the field depuration presented in this paper. Mussels from the same long-line farm with similar initial OA levels were used and the experiment started ten days prior to the current field experiment. Thus, the physiological status and toxin history of the mussels can be considered equal and comparisons of depuration rates between these experiments are therefore relevant. The results showed that depuration was equally effective regardless of external factors (temperature and food conditions) with a calculated half-life of eight days for all treatments. This rate was very similar to the field depuration rate observed in this present study ($t_{1/2}$=7 days). Other studies where attempts have been made to accelerate the depuration process in *M. edulis* by changing the conditions for the mussels have also shown that rates of depuration of DST remain unaffected (Lindahl & Hageltorn, 1986; Svensson, 2002). These results point to the difficulties incurred in attempts to influence the rate of depuration of DST by manipulating exogenous factors (Morono *et al.*, 1998). Seasonal changes in the physiological status and annual reproductive cycle, especially in mussels from temperate latitudes, may contribute to the variability in depuration rates of DST observed in both the field and laboratory (Svensson & Förlin, in prep.). Indeed, slow depuration rates were observed in Swedish mussels by Svensson (2003) and Lindahl & Hageltorn (1986) during November to March whereas faster rates were reported during late summer in this paper and by Svensson & Förlin (in prep.). Thus, season should be considered a relevant factor to include when uptake and elimination of toxins are studied.

Using data on several plankton variables, additional information about the potential causative factors determining toxicity levels in the mussels could be extracted. However, interpretation of relationships between plankton data, sampled once a week, and mussel toxicity, which is a time-integrated measure and reflects previous situations in the water column, is always problematic. This has to be accounted for when conclusions are drawn. Total plankton biomass was used as a measure of food available for the mussels at both locations. The importance of feeding physiology for depuration of DST in mussels have been discussed in previous papers (Sampayo *et al.*, 1990; Marcaillou-Le Baut *et al.*, 1993; Blanco *et al.*, 1995; Blanco *et al.*, 1999; Svensson, 2003). It has been suggested that both the total biomass of plankton as well as the relative proportion of *Dinophysis* in the plankton community could be factors critical for depuration. These factors are likely to affect the filtration and uptake rates of toxic algae in the mussels. A reduced ingestion rate of toxic *Dinophysis* will lead to a reduction in the DST concentration even though the elimination rate remains constant. No significant difference in plankton biomass was found during this experiment, thus this

variable did not account for the difference in OA concentration observed between farm and fjord mussels. The proportion of both *D. acuminata* and *D. acuta* varied between and within locations during the experiment which could have affected the ingestion rate of these species and hence levels of OA in mussels. However, this mechanism is not important if the algal species do not produce OA, or the toxicity per cell is highly variable which appeared to be the case in our study. For example, there were no correlations between cell numbers and plankton toxicity in the farm. Even so, toxicity was detected in all plankton samples, indicating that mussels continued to ingest OA-producing algae in the farm. In contrast to the farm site, we did not detect plankton toxicity during the first two sampling occasions in the fjord. This absence of plankton toxicity probably caused the initial fast reduction of OA in the experimental mussels. Highly variable toxin contents per cell for both *D. acuta* and *D. acuminata* have also been confirmed in Denmark, Norway, Spain and France (Andersen *et al.*, 1996; Aune *et al.*, 1996; Dahl & Johannessen, 2001; Fernández *et al.*, 2002; Marcaillou *et al.*, 2002). This complicates the use of absolute cell counts, or proportion *Dinophysis*, as predictive measures of mussel toxicity (Fernández *et al.*, 2002). A better predictive variable would be the factual toxin concentration in plankton extracts (Godhe *et al.*, 2002).

Mussels depurated from OA were unsuitable for consumption at the end of the experiment due to the accumulation of DTX-1. *D. acuta* was confirmed to be the causative species for DTX-1 in the fjord area. The rapid accumulation of DTX-1 observed in fjord mussels between the last sampling occasions correlated to high numbers of *D. acuta* as well as high plankton toxicity in the fjord. Since *D. acuminata* has no previous history of DTX-1 (Yasumoto *et al.*, 1985; Andersen *et al.*, 1996; Vale & Sampayo, 2000; Fernández *et al.*, 2002; Marcaillou *et al.*, 2002) and *D. norvegica* is regarded as a low, or non-toxic species in Scandinavian waters (Dahl & Johannessen, 2001; Andersen *et al.*, 1996), this further connects *D. acuta* to DTX-1 production. Interestingly, the same species did not appear to produce DTX-1 in the coastal farm location. DTX-1 was never detected in farm mussels even though *D. acuta* was present in all plankton samples. Thus, two important conclusions about the fjord system can be drawn: (1). This area is not always safe for depuration of DST in mussels. (2). Different toxin profiles in *D. acuta* between the farm site and the fjord suggest that the two populations are genetically distinct and/or that differences in environmental conditions affect the production of DST components.

Several trials to detoxify shellfish containing paralytic shellfish toxins (PST) have been done (see reviews by (Bricelj & Shumway, 1998) and (Shumway *et al.*, 1995). Methods testing temperature and salinity stress, chlorination and ozone treatment of the seawater are reported but none has been shown to be effective in accelerating the rate of detoxification of PST. As for DST, the rate of depuration of PST appears to change with the season where slower rates are recorded during colder periods.

To conclude, the most cost-effective depuration for DST will be achieved during conditions when mussels are self-depurated in waters free of toxin-producing dinoflagellates, possibly with a faster reduction rate during the warmer season and vice versa.

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References

Andersen, P. 1996. Design and implementation of some harmful algal monitoring systems. IOC Technical Series no. 44. UNESCO, Paris. 102 pp.

Andersen, P., Hald, B., Emsholm, H., 1996. Toxicity of *Dinophysis acuminata* in Danish coastal waters. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 281-284

Aune, T., Strand, Ö., Aase, B., Weidemann, J., Dahl, E., Hovgaard, P., 1996. The Sognefjord in Norway, a possible location for mussel farming? In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 73-76

Blanco, J., Fernández, M., Marino, J., Reguera, B., Míguez, A., Maneiro, J., Cacho, E., Martínez, A., 1995. From *Dinophysis* spp. toxicity to DSP outbreaks: a preliminary model of toxin accumulation in mussels. In: Lassus, P., Arzul G., Erard E., Gentien P., Marcaillou C., (Ed.), Harmful marine algal blooms. Lavoisier, Intercept Ldt, Paris, pp. 777-782

Blanco, J., Fernández, M. L., Míguez, A., Morono, A., 1999. Okadaic acid depuration in the mussel Mytilus galloprovincialis: one- and two-compartment models and the effect of environmental conditions. Mar. Ecol. Prog. Ser. 176, 153-163

Bricelj, M. V., Shumway, S. E., 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics and biotransformation. Reviews in Fisheries Science 6 (4), 315-383

Croci, L., Toti, L., De Medici, D., Cozzi, L., 1994. Diarrhetic shellfish poisoning in mussels: comparison of methods of detection and determination of the effectiveness of depuration. J. Int. Food Microbiol. 24, 337-342

Dahl, E., Johannessen, T., 2001. Relationship between occurrence of Dinophysis species (Dinophyceae) and shellfish toxicity. Phycologia 40 (3), 223-227

Edebo, L., Lange, S., Li, X.P., Allenmark, S., Lindgren, K., Thompson, R., 1988. Seasonal, geographic and individual variation of okadaic acid content in cultivated mussels in Sweden. APMIS 96, 1036-1042

Edebo, L., Haamer, J., Lange, S., Li, X.P., 1991. Okadaic acid in blue mussels in Sweden diminishes with the distance from the open sea. Revue Internationale d´Oceanographie Medicale 101-104, 172-175

Edler, L. 1977. Phytoplankton and primary production in the Sound. Ph D Thesis, Dept. of Marine Botany, Göteborg University, Göteborg.

Fernández, M. L., Reguera, B., Ramilo, I., Martínez, A. 2002. Toxin content of *Dinophysis acuminata*, *D. acuta* and *D. caudata* from the Galician rias bajas. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J., Lewis, R.J., Harmful algal blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 360-363.

Godhe, A., Svensson, S., Rehnstam-Holm, A.-S., 2002. Oceanographic settings explain fluctuations in *Dinophysis* spp. and concentrations of Diarrhetic Shellfish Toxin in the plankton community within a mussel farm area on the Swedish west coast. Mar. Ecol. Prog. Ser. 240, 71-83

Haamer, J., Andersson, P-O., Lange, S., Li, X.P., Edebo, L., 1990a. Effects of transplantation and reimmersion of mussels Mytilus edulis L. on their

contents of okadaic acid. J. Shellfish Res. 9 (1), 109-112

Haamer, J., Andersson, P-O., Lindahl, O., Lange, S., Li, X.P., Edebo, L., 1990b. Geographic and seasonal variation of okadaic acid content in farmed mussels, Mytilus edulis L., along the Swedish west coast. J. Shellfish Res. 9 (1), 103-108

Haamer, J., 1995. Presence of the phycotoxin okadaic acid in mussels (*Mytilus edulis*) in relation to nutrient composition in a Swedish coastal water. J. Shellfish Res. 14 (1), 209-216

Hallegraeff, G. M., 1995. Harmful algal blooms: a global overview. In: Hallegraeff, G. M., Anderson D. M., Cembella A., (Ed.), Manual on harmful marine microalgae. UNESCO, Paris, pp. 1-22

Hansen, G., 1992. Biomasseberegningar. In: Thomsen, H. A., (Ed.), Plankton i de indre danske farvand. Miljostyrelsen, Copenhagen, pp. 20-34 (In Danish)

Kollberg, S. 1999. Beskrivning av den svenska musselnäringen. Vattenbrukarnas Riksförbund, 68 pp (In Swedish).

Krogh, P., Edler, L., Graneli, E., Nyman, U., 1985. Outbreak of Diarrhetic Shellfish Poisoning on the west coast of Sweden. In: Anderson, D.A., White, Baden., (Ed.), Toxic Dinoflagellates. Elsevier Science Publishing, Amsterdam, pp. 501-503

Kumagai, M., Yanagi, T., Murata, M., Yasumoto, T., Kat, M., Lassus, P., Rodriguez-Vazquez, J.A., 1986. Okadaic acid as the causative toxin of diarrhetic shellfish poisoning in Europe. Agric. Biol. Chem. 50 (11), 2853-2857

Lassus, P., Proniewski, F., Maggi, P., Truquet, P., Bardouil, M. 1993. Wind-induced toxic blooms of *Dinophysis* cf. *acuminata* in the Antifer area. In: Smayda, T.J., Shimizu, Y (Eds.).Toxic phytoplankton blooms in the sea. Elsevier Science, New York. 519-523.

Lee, J. S., Yanagi, T., Kenma, R., Yasumoto, T., 1987. Fluorometric determination of diarrhetic shellfish toxins by high pressure liquid chromatography. Agric. Biol. Chem. 51, 877-881

Lindahl, O., Andersson, B., 1996. Environmental factors regulating the occurrence of *Dinophysis* spp. in the Koljö fjord, Sweden. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 269-272

Lindahl, O., Hageltorn, M. 1986. Detoxification experiments of DSP in blue mussels. 15th Nordic Veterinary Congress. 463-466.

Lindegarth, M. 1997. Sammanställning och tolkning av de kontinuerliga mätningarna av okadasyrahalter längs den svenska västkusten. Länsstyrelsen Västra Götaland, 17 pp. (In Swedish).

Marcaillou, C., Gentien, P., Lunven, M., Le Grand, J., Mondeguer, F., Danilou, M. M., Crassous, M. P. *et al*. 2000. *Dinophysis acuminata* distribution and specific toxin content in relation to mussel contamination. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J., Lewis, R.J., Harmful algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 356-359.

Marcaillou-Le Baut, C., Bardin, B., Bardouil, M., Bohec, M., Le Dean, L., Masselin, P., Truquet, P., 1993. DSP depuration rates of mussels reared in a laboratory and an aquaculture pond. In: Smayda, T. J., Shimizu Y., (Ed.), Toxic phytoplankton blooms in the sea. Elsevier Science Publisher, pp. 531-535

Morono, A., Fernández, L. M., Franco, J. M., Martínez, A., Reyero, I. M., Míguez, A., Cacho, E., Blanco, J., 1998. PSP and DSP kinetics in mussel, *Mytilus galloprovincialis*: effect of environmental parameters and body weight. In: Reguera, B., Blanco J., Fernández M. L., Wyatt T., (Ed.), Harmful Algae. Xunta de Galicia

and Intergovernmental Oceanographic Commission of UNESCO, pp. 445-448

Poletti, R., Viviani, R., Casadei, C., Lucentini, L., Funari, E., Draisci, R., 1996. Decontamination dynamics of mussels naturally contaminated with diarrhetic toxins relocated to a basin of the Adriatic sea. In: Yasumoto, T., Oshima Y., Fukuyo Y., (Ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 429-432

Ramstad, H., Hovgaard, P., Yasumoto, T., Larsen, S., Aune, T., 2001. Monthly variations in diarrhetic toxins and yessotoxin in shellfish from coast to the inner part of the Sognefjord, Norway. Toxicon 39 (7), 1035-1043

Reguera, B., Bravo, I., Fraga, S., 1995. Autoecology and some life history stages of *Dinophysis acuta* Ehrenberg. J. Plankton Research 17, (5), 999-1015

Sampayo, M. A., Alvito, P., Franca, S., Sousa, I., 1990. *Dinophysis* spp. toxicity and relation to accompanying species. In: Granéli, E., Sundström B., Edler L., Anderson D. M. M., (Ed.), Toxic marine phytoplankton. Elsevier, New York, pp. 215-220

Sedmak, B., Fanuko, N., 1991. Occurence of *Dinophysis* spp. and toxic shellfish in the Northern Adriatic. J. Appl. Phyc. 3, 289-294

Shumway, S. E., van Egmond, H. P., Hurst, J. W., Bean, L. L., 1995. Management of shellfish resources. In: Hallegraeff, G. M., Anderson D. M., Cembella A., (Ed.), Manual on harmful marine microalgae. UNESCO, Paris, pp. 433-462

Svensson, S., 2003. Depuration of okadaic acid (diarrhetic shellfish toxin) in mussels, *Mytilus edulis* (Linnaeus), feeding on different quantities of non-toxic algae. Aquaculture 218, 277-291

Svensson, S., Förlin, L. (in prep.). Analysis of the relationship between lipid content and depuration of okadaic acid (diarrhetic shellfish toxin) in mussels, *Mytilus edulis*: results from a field study and a laboratory experiment.

Underdal, B., Yndestad, M., Aune, T., 1985. DSP intoxication in Norway and Sweden, autumn 1984-spring 1985. In: Anderson, D.A., White, Baden, (Ed.), Toxic Dinoflagellates. Elsevier Science Publishing, Amsterdam, pp. 489-494

Vale, P., Sampayo, M. A. d. M., 2000. Dinophysistoxin-2: a rare diarrhoeic toxin associated with *Dinophysis acuta*. Toxicon 38, 1599-1606

Vieites, J. M., Leira Sanmartin, F., 2000. Incidence of marine toxins on industrial activity. In: Botana, L. M., (Ed.), Seafood and freshwater toxins. Pharmacology, physiology and detection. Marcel Dekker, New York, pp. 741-760

Vieytes, M. R., Fontal, O. I., Leira, F., Baptista de Sousa, J. M. V., Botana, L. M., 1997. A fluorescent microplate assay for diarrheic shellfish toxins. Anal. Biochem. 248 258-264

Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G. K., Clardy, J., 1985. Diarrhetic shellfish toxins. Tetrahedron 41, 1019-1025

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A CASE OF CONSISTENT SPATIAL DIFFERENCES IN CONTENT OF DIARRHETIC SHELLFISH TOXINS (DST) AMONG THREE BIVALVE SPECIES: *MYTILUS EDULIS*, *OSTREA EDULIS*, AND *CERASTODERMA EDULE*

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ABSTRACT Content of diarrhetic shellfish toxins (DST) was compared among mussels (*Mytilus edulis*), oysters (*Ostrea edulis*), and cockles (*Cerastoderma edule*) at two spatial scales: regions (100 km apart) and locations within regions (5 km apart). Samples were analysed for DST using protein phosphatase inhibiton assay in individual digestive glands. Concentrations of DST in all oysters and cockles were below the detection limit in the assay, whereas mussels from both regions and all locations contained mean levels of DST above the regulation limit for harvest and marketing. Thus interspecific differences in content of DST were found along the Swedish west coast. Some behavioral and physiological phenomena are proposed to explain the differences among species. These include differential uptake and processing of toxic algae, biotransformation of toxins, and reduced filtration at low temperatures. These findings may have some implications for harvest and cultivation of bivalves and suggest a possibility that cockles and oysters could be marketed for human consumption during periods of elevated levels of DST in mussels.

KEY WORDS: *Cerastoderma edule*, cockles, diarrhetic shellfish toxins, DST, interspecific differences, *Mytilus edulis*, mussels, okadaic acid, OA, *Ostrea edulis*, oysters, protein phosphatase inhibition assay

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is one of several illnesses caused by the consumption of shellfish containing toxic substances produced by marine microalgae. The most common toxins associated with DSP belong to the okadaic acid (OA) group (Yasumoto et al. 1985). DSP was first described in Japan during the late 1970s, but has since been reported from temperate waters around all continents where it causes considerable problems for harvesters and consumers of shellfish.

In Sweden, monitoring for DSP toxins (DST) in blue mussels, *Mytilus edulis*, has been ongoing since 1988. This has revealed large seasonal, geographical, and individual variations in content of DST in mussels along the West Coast (Lindegarth 1997). For example, mussels generally contain DST above the quarantine levels (160 μg OA kg^{-1} mussel meat, EC regulations) for harvest during the autumn and winter period, sometimes for up to 6 mo each year.

DST is the most serious threat to a sustainable industry based on cultured blue mussels in Sweden and there is a need for managerial actions to reduce its impact. Aquaculture and fisheries for a more diverse range of bivalve species, which may vary in content of toxins, could be an alternative during periods of high levels of DST in mussels. As an example, the knowledge about interspecific differences in levels of paralytic shellfish toxins (PST) are used by authorities in Maine, who have been practising species-specific harvest and closure of bivalve fisheries for many years (Dr. S. Shumway pers. comm.). In Sweden, small-scale fisheries for the European oyster, *Ostrea edulis*, and cockles, *Cerastoderma edule* and *Ceratoderma lamarcki*, are in operation today. These species are currently not included in DST monitoring and information about the presence of DST in oysters and cockles is sparse.

As mentioned earlier, differential patterns of accumulation and depuration of PST among species of bivalves have been observed (Bricelj and Shumway 1998). In general, *Mytilus* sp. rapidly accumulate and detoxify PST compared to most other species. Some observations on differences in content of DST among species of bivalves collected in the field have also been made (MacKenzie et al. 1998, Poletti et al. 1998). *Mytilus* spp. were reported to contain higher levels of DST compared to the other species included in these studies. However, these studies were not specifically performed to investigate differences among species concerning their ability to accumulate DST. For that purpose shellfish should be collected at the same time and habitat to reduce effects of differences in exposure to toxic algae prior to sampling.

During the summer of 1998, high levels of OA (>1 mg OA kg^{-1} mussel meat) were detected in blue mussels from a farm located in north Bohuslän on the Swedish west coast. At the same time, we

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observed that oysters and cockles, collected from the vicinity of the farm, contained non-detectable levels of DST measured by HPLC. To test whether the observed differences in DST among these species were real and consistent in space, we sampled at two different spatial scales. This paper reports the results from this study.

MATERIALS AND METHODS

Naturally occurring cockles (*Cerastoderma edule*), oysters (*Ostrea edulis*), and mussels (*Mytilus edulis*) were collected during 1 wk in late November to early December 1998 from two regions separated by approximately 100 km along the coast of Bohuslän County, Sweden (A: Ljungskile and B: Tjärnö, Fig. 1). In these regions mussels are commercially farmed and fishing for both cockles and oysters occurs. Sampling was performed when levels of OA in farmed mussels had been reported to be high for more than 2 mo in both areas (OA data from the weekly monitoring program). Within each region sampling was done at each of two locations, separated by approximately 5 km (Buvenäs and Sparreviken in region A, Tenskär and Kockholmen in region B, Fig. 1). These locations were selected because all three species were found to co-exist on a small scale. Six individuals of each species were taken from each location at depths between 0.5 and 3 m within a radius of 20 m. Sampling depths and size characteristics of the samples from each location are shown in Table 1. Water temperatures were between 3 °C and 5 °C at the time of collection. The specimens were frozen and stored at −20 °C until toxin analysis was performed.

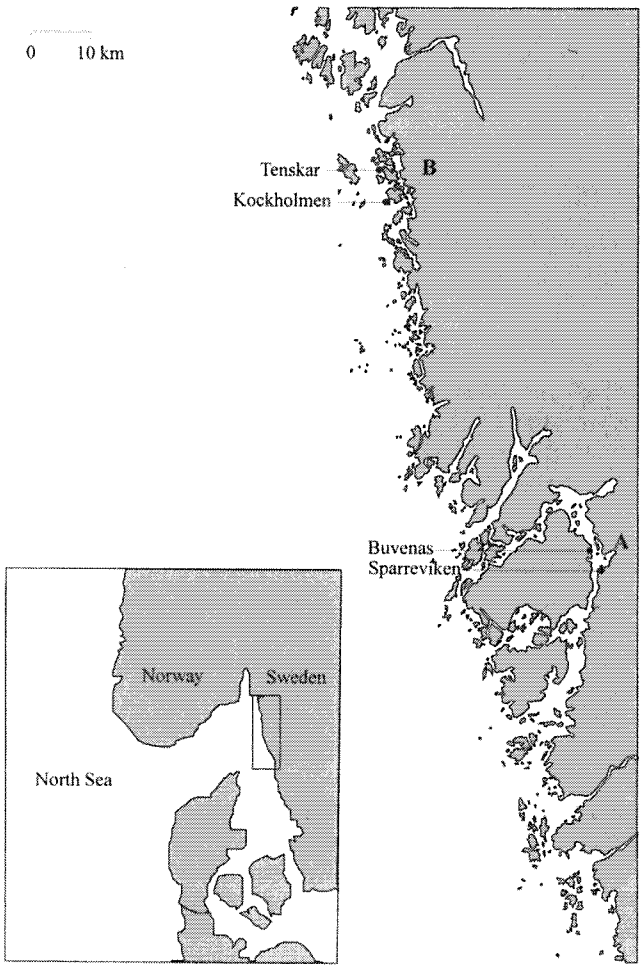

Figure 1. Map of the coastal area of Bohuslän County showing the sampling locations of *M. edulis*, *O. edulis*, and *C. edule*. (A) Ljungskile region. (B) Tjärnö region.

Preparations of shellfish extracts were done on individual digestive glands using the sample clean-up protocol according to Lee et al. (1987). Concentrations of DST in the resulting chloroform extracts were then analyzed using the fluorescent microplate phosphatase inhibition assay (PIA) according to Vieytes et al. (1997) with some modifications which will be published elsewhere (Rehnstam-Holm et al. in prep.). Toxin content was expressed as micrograms of OA equivalents g^{-1} of digestive gland and micrograms of OA equivalents kg^{-1} mussel meat. The detection limit for shellfish extracts was 5 ng OA equivalents g^{-1} digestive gland when dilution factors were considered.

To confirm the accuracy of the PIA method, 4 individuals of each species were chosen at random and analyzed for OA and DTX-1 by HPLC according to Lee et al. (1987) using 1-pyrenyldiazomethan (PDAM) instead of ADAM.

RESULTS

Results from the PIA analyses are shown in Table 1. All the cockle and oyster samples contained levels of DST below the detection limit for the PIA, whereas toxins were detected in all individual mussels. Mean concentration in mussels varied between 1.5 to 2.6 μg OA equivalent g^{-1} digestive gland or 209 to 241 μg OA equivalent kg^{-1} mussel meat which is above the regulation limit for marketing of mussels.

When analysed by HPLC, neither OA nor DTX-1 were detected in the oyster and cockle extracts, whereas OA was detected in all mussel samples. Low amounts of DTX-1 compared to OA (>10%) were found in two of the mussel samples. OA equivalent measured by the PIA method correlated well to concentrations of OA in the HPLC method (data not shown). Thus HPLC confirmed the results from the PIA that OA and DTX-1 were absent (non-detectable) in the oyster and cockle samples.

This study was designed to test hypotheses about interspecific and spatial variability in DST using multifactorial ANOVA. Since no toxins were detected in *C. edule* and *O. edulis*, tests with normal parametric procedures could not be justified in order to test hypotheses about interspecific differences in mean content of DST. Nevertheless, confidence intervals did not include the value for detection limit for the PIA which indicated that the sample size (n = 6) was sufficiently large to allow unambiguous conclusions about interspecific differences.

DISCUSSION

The purpose of this study was to investigate interspecific and spatial variability of DST in 3 bivalve species that co-exist in Swedish waters. To our knowledge this is the first study where levels of DST have been compared among bivalve species that were sampled from the same locations under similar conditions in the field. The consistent differences in levels of DST found between blue mussels on one hand and cockles and oysters on the other hand provided evidence for interspecific differences that are not caused by differences in exposure to toxic algae. Thus the pattern that was found indicated differences in either the ability to accumulate or depurate DST among the species studied.

TABLE 1.

Sampling depths, size characteristics, and content of OA, analyzed by PIA in *C. edule, M. edulis,* and *O. edulis* from the 4 study locations. % d.g.: proportion digestive gland (%) of total tissue wet weight. Values for size characteristics are means ± SD (n = 6). Concentration of OA is expressed as µg OA equiv. g^{-1} digestive gland and µg OA equiv. kg^{-1} mussel meat. n.d.: no toxins detected (detection limit 5 ng OA equiv. g^{-1} digestive gland). Values for OA are means ± 95% confidence intervals (n = 6).

Species	Region	Location	Depth (m)	Shell length (mm)	Tissue wet weight (g)	% d.g.	OA equiv. (µg g^{-1})	OA equiv. (µg kg^{-1})
C. edule	Ljungskile	Buvenäs	0.5–0.7	40.0 ± 5.7	10.4 ± 4.3	7.6 ± 1.2	n.d.	
		Sparreviken	0.5	44.5 ± 4.6	13.0 ± 2.5	8.4 ± 1.8	n.d.	
	Tjärnö	Kockholmen	0.5–0.7	40.5 ± 2.7	9.3 ± 1.8	11.5 ± 3.2	n.d.	
		Tenskär	0.6–1.0	26.8 ± 9.2	3.4 ± 2.7	11.4 ± 1.4	n.d.	
M. edulis	Ljungskile	Buvenäs	0.5–0.7	63.2 ± 7.9	17.5 ± 7.0	8.6 ± 1.8	2.6 ± 1.4	222 ± 119
		Sparreviken	3.0	73.5 ± 7.2	29.9 ± 9.0	9.6 ± 2.0	2.4 ± 2.0	242 ± 223
	Tjärnö	Kockholmen	1.7–2.2	74.5 ± 14.7	29.2 ± 14.3	14.8 ± 3.2	1.4 ± 0.5	209 ± 92
		Tenskär	0.6–1.0	57.3 ± 14.3	16.6 ± 11.2	13.4 ± 2.9	1.5 ± 0.5	210 ± 105
O. edulis	Ljungskile	Buvenäs	0.5–0.7	71.3 ± 13.0	10.8 ± 6.1	9.2 ± 2.7	n.d.	
		Sparreviken	3.0	80.3 ± 15.5	20.0 ± 10.4	9.1 ± 2.2	n.d.	
	Tjärnö	Kockholmen	1.7–22	91.5 ± 8.8	19.7 ± 8.5	8.2 ± 1.8	n.d.	
		Tenskär	0.6–1.0	54.3 ± 5.9	2.7 ± 1.2	11.3 ± 3.6	n.d.	

To explain the interspecific differences in content of DST, some behavioral and physiological phenomena can be proposed as discussed below.

Interspecific variability in pre- and/or post-ingestive selection may occur in order to increase or reduce the uptake and processing of the DST-producing algae. *O. edulis* from the North American east coast has been found to selectively clear both toxic (PST) and non-toxic dinoflagellates from mixed cell suspensions in laboratory experiments (Shumway and Cucci 1987). Sidari et al. (1998) observed that during an event of DSP in Italy, *M. galloprovincialis* seemed to feed selectively on *Dinophysis* sp., comparing algal contents in the stomachs to those of the water column. Although there are no previous observations on selection against algae containing DST, a possible explanation of our results is that the sampled populations of *O. edulis* and *C. edule* may be rejecting these species, either pre- or post-ingestion, thus rendering the non-toxic results in the analysis.

Recent evidence suggests that DST can be biotransformed by bivalves (Lee et al., 1989, Suzuki et al. 1999, Fernández et al. 1996). A group of low-polar acyl-ester derivatives of OA, DTX-1 (referred to as DTX-3), and DTX-2 has been found in shellfish, but never in the dinoflagellates producing DST. Therefore, it has been suggested that the acylated forms of DST are products of metabolic activity in the digestive glands of the molluscs (Lee et al. 1989). Using modifications of the HPLC method, Suzuki et al. (1999) reported that Japanese scallops, *Pactinopecten yessoensis,* rapidly converted DTX-1 to DTX-3 with significantly higher content of the latter in the tissue. In contrast, Fernández et al. (1996) found that although mussels, *M. galloprovincialis,* contained detectable amounts of acylated DST, the major content in the extracts was always OA, implying only a slow rate of biotransformation of OA for mussels. Due to the chemical nature of the acyl derivatives, they are not detected using traditional clean-up procedures for HPLC. Also, the sensitivity of the PIA method to DTX-3 are low compared to OA and DTX-1 (Mountfort et al. 1999). Thus the absence of OA and DTX-1 in oyster and cockle extracts in our study could be explained by a rapid acylation of these compounds in the digestive glands, which in that case, we were unable to detect by PIA and HPLC. In future studies it should be possible to test this hypothesis by using modifications of the HPLC method. It is also possible that rates of depuration of the non-acylated compounds vary among species, which could be evaluated by performing depuration experiments.

The interspecific differences in content of DST could also be an effect of water temperature since sampling was performed during a time of the year when temperatures were low (3 °C–5 °C). This is close to the temperature when filtration is reduced or inhibited in oysters (Child and Laing 1998). Mussels, on the other hand, are active even at temperatures close to 0 °C (Loo 1992). Reduced filtration and thus uptake of toxic algae in oysters and also cockles may therefore explain the results. Also, in case that these species did contain toxins earlier during the season, depuration may have occurred after filtration (uptake of toxic algae) stopped.

Although the mechanisms causing the interspecific differences in content of DST remain unknown, the observations made in this study could have important implications for harvest and cultivation bivalves. Currently within the EC, marketing of shellfish from certain areas is regulated by content of algal toxins in blue mussels, a species which generally accumulates high levels of toxins. Our results suggest the possibility that oysters and cockles could be harvested and marketed for human consumption during periods when mussels contain significant levels of toxins. However, further sampling and analysis of DST in oysters and cockles, including temporal replication must be performed during periods of DST in order to confirm the results found in this study. Also, biological tests should be conducted in parallel with the chemical analyses to test whether any toxicity due to unknown substances or biotransformed products is present in the shellfish meat.

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LITERATURE CITED

Bricelj, M.V. & S.E. Shumway. 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics and biotransformation. *Rev Fisheries Sci.* 6:315–383.

Child, A.R. & I. Laing. 1998. Comparative low temperature tolerance of small juvenile European oyster, *Ostrea edulis* L., and Pacific oyster, *Crassostrea gigas* Thunberg. *Aquacult. Res.* 29:103–113.

Fernández, M.L., A. Míguez, E. Cacho & A. Martínez. 1996. Detection of okadaic acid esters in the hexane extracts of Spanish mussels. *Toxicon* 34:381–387.

Lee, J.S., T. Yanagi, R. Kenma & T. Yasumoto. 1987. Fluorometric determination of diarrhetic shellfish toxins by high pressure liquid chromatography. *Agric. Biol. Chem.* 51:877–881.

Lee, J.S., T. Igarashi, S. Fraga, E. Dahl, P. Hovgaard & T. Yasumoto. 1989. Determination of diarrhetic shellfish toxins in various dinoflagellate species. *J. Appl. Phycol.* 1:147–152.

Lindegarth, M. 1997. Sammanställning och tolkning av de kontinuerliga mätningarna av okadasyrahalter längs den svenska västkusten. Bohuskustens Vattenvårdsförbund, Göteborg (In Swedish).

Loo, L.-O. 1992. Filtration, assimilation, respiration and growth of *Mytilius edulis* at low temperatures. *Ophelia* 35:123–131.

MacKenzie, L., P. Truman, M. Satake, T. Yasumoto, J. Adamson, D. Mountfort & D. White. 1998. Dinoflagellate blooms and associated DSP-toxicity in shellfish in New Zealand. pp. 74-77. *In*: B. Reguera, J. Blanco, M.L . Fernández, and T. Wyatt (eds.) .Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.

Mountfort, D.O., G. Kennedy, I. Garthwaite, M. Quilliam, P. Truman & D. J. Hannah. 1999. Evaluation of the fluorometric protein phosphatase inhibition assay in the determination of okadaic acid in mussels. *Toxicon.* 37:909–922.

Poletti, R., K. Cettul, F. Bovo, A. Milandri, M. Pompei & R. Frate. 1998. Distribution of toxic dinoflagellates and their impact on shellfish along the northwest Adriatic coast. pp. 88-90. *In*: B. Reguera, J. Blanco, M.L. Fernández, and T. Wyatt (eds.). Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.

Shumway, S.E. & T. L. Cucci. 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. *Aquat. Toxicol.* 10:9–27.

Sidari, L., P. Nichetto, S. Cok, S. Sosa, A. Tubaro, G. Honsell & R. Della Loggia. 1998. Phytoplankton selection by mussels, and diarrhetic shellfish poisoning. *Mar. Biol.* 131:103–111.

Suzuki, T., H. Ota & M. Yamasaki. 1999. Direct evidence of transformation of dinophysistoxin-1 to 7-acyl-dinophysistoxin-1 (dinophysistoxin-3) in the scallop *Pactinopecten yessoensis*. *Toxicon.* 37:187–198.

Vieytes, M.R., O. I. Fontal, F. Leira, J. M. V. Baptista de Sousa & L. M. Botana. 1997. A fluorescent microplate assay for diarrheic shellfish toxins. *Anal. Biochem.* 248:258–264.

Yasumoto, T., M. Murata, Y. Oshima, M. Sano, G. K. Matsumoto & J. Clardy. 1985. Diarrhetic shellfish toxins. *Tetrahedron* 41:1019–1025.

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