

The stress protein HSP70 from the marine sponge *Thenea muricata*

K. VALLMANN¹, C. KIVISILD¹, A. LOPP¹, H.T. RAPP² AND M. KELVE¹

¹Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, Tallinn 12618, Estonia, ²Department of Biology and Centre for Geobiology, University of Bergen, Thormøhlensgate 53A, N-5020 Bergen, Norway

*The members of the heat shock protein 70 (HSP70) family are among the most conserved and widely studied stress proteins. The transcription and translation levels of HSP70 genes have also been studied in several marine and freshwater sponges as molecular markers to characterize the response of sponges to various types of physiological or environmental stress conditions. Furthermore, HSP70 protein sequences have been used in phylogenetic analysis of prokaryotes and eukaryotes. In this study, the expression of HSP70 genes in the marine sponge *Thenea muricata* during long-term cultivation under laboratory conditions was described at the protein and mRNA levels. Though there are many studies about distribution and morphology of *T. muricata*, few biochemical and molecular data can be found in the literature. HSP70 gene data for several sponge species have been deposited in the NCBI database, however, those for *T. muricata* are not available. Therefore, HSP70 genes were characterized in this sponge. Several proteins of the HSP70 superfamily which might be induced by stress, were present in *T. muricata*.*

Keywords: Environmental stress, HSP70, *Thenea muricata*, marine sponge, long-term cultivation

Submitted 30 September 2014; accepted 10 March 2015; first published online 13 January 2016

INTRODUCTION

The members of the heat shock protein 70 (HSP70) family are among the most conserved and widely studied stress proteins. Due to their high abundance and high conservation of their primary structures and functions, they are also widely used in phylogenetic analysis. For example, sequences of HSP70s have been used to confirm monophyly of Metazoa (Borchiellini *et al.*, 1998).

The HSP70 is a large multigene protein family with members residing in a number of subcellular compartments, including the cytoplasm, mitochondria and endoplasmic reticulum (ER). Members of the HSP70 family assist in a large variety of cellular processes to ensure cellular homeostasis during normal and stressful conditions. The functions of HSP70s include the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of organellar and secretory proteins, and control of the activity of regulatory proteins (Mayer & Bukau, 2005). Heat shock proteins participate in minimizing the aggregation of non-native proteins and targeting non-native or aggregated proteins for degradation and removal from the cell (Feder & Hofmann, 1999). The broad range of functions has been achieved through the amplification and diversification of HSP70 genes in evolution (generating specialized HSP70 chaperones), a special range of co-chaperones that target HSP70s to their substrates, and cooperation with other chaperone systems (Mayer & Bukau, 2005).

The need for molecular chaperones is accelerated under stressful conditions that could potentially damage the cellular and molecular structures in the cell (Buchberger *et al.*, 2010). Factors of both anthropogenic and natural origin have been shown to induce the synthesis of HSPs in organisms ranging from bacteria and plants to mammals (Sanders, 1993). In marine organisms, factors that have all been linked to stress comprise those of environmental origin, as well as those caused by anthropogenic pollution, introduced species and nutrient enrichment (Lafferty *et al.*, 2004).

The HSP70s are among the most conserved proteins, having 60–78% identity of amino acid sequences among eukaryotic organisms and 40–60% identity among eukaryotic HSP70s and *Escherichia coli* DnaK sequence (Bardwell & Craig, 1984; Kregel, 2002).

Eukaryotic genomes have multiple HSP70 genes including the genes that are solely inducible, those constitutively expressed but inducible by stress, and those solely constitutively expressed (heat shock cognates) (Hendrick & Hartl, 1993). The human genome harbours at least 17 genes coding for proteins of the HSP70 family (Brocchieri *et al.*, 2008). Glucose regulated proteins GRP75 and GRP78 that also have different levels of inducibility, form a separate group among the HSP70 protein family. Glucose regulated proteins are stress-inducible chaperones that mostly reside in the ER or mitochondria. They are constantly expressed at basal level and elevated levels of GRP78 are used as indicators for ER stress. Glucose regulated protein 78 takes part in a vast number of physiological processes: the stress response, proliferation, apoptosis, angiogenesis, inflammation and immune response (Lee, 2014).

The use of HSPs as biomarkers is most widely used in aquatic toxicology (Feder & Hofmann, 1999). The sessile lifestyle of sponges and their constant filtering of surrounding

Corresponding author:
K. Vallmann
Email: kerli.vallmann@gmail.com

water, with their ability to respond to environmental stress, makes them especially suitable as biomarkers to assess the effects of pollution (Agell *et al.*, 2001; Cebrian *et al.*, 2006). It has been shown that in addition to heat stress, the increase in HSP70 levels can also be induced by a number of factors including heavy metals (Agell *et al.*, 2001), cold (Kozioł *et al.*, 1996), acoustic stress (Filiciotto *et al.*, 2014), and space competition or aggression among sessile marine invertebrates (Rossi & Snyder, 2001). Recently HSP70s have reattracted a lot of attention as markers for heat stress in the light of climate warming (Fan *et al.*, 2013; Webster *et al.*, 2013).

The first documentation of HSP response in sponges goes back to 1993 when elevated immunologically detectable HSP70 levels were seen in cubes of the marine sponge *Geodia cydonium* that were treated with tributyltin in laboratory conditions (Batel *et al.*, 1993). A significant increase in immunologically detectable HSP70 levels was observed in that sponge in response to temperature and pH changes (Kozioł *et al.*, 1996). Kozioł *et al.* (1996) cloned the first cytosolic putatively inducible HSP70 cDNA from *G. cydonium*. In an attempt to solve the deep phylogeny of sponges, cytosolic HSP70s were also cloned from *Sycon raphanus*, the marine sponge that belongs to the class Calcarea, and *Rhabdocalyptus (Acanthascus) dawsoni*, the marine sponge that belongs to the class Hexactinellida (Kozioł *et al.*, 1997). In 1998, ER protein GRP78 from *Suberites domuncula* was added to the cloned HSP70 family proteins from sponges (Kozioł *et al.*, 1998).

The expression of proteins from the HSP70 superfamily has been studied in several sponge species in different stressful conditions from heat and pollutants to disease-like conditions as well as through developmental processes of a freshwater sponge (Müller *et al.*, 1995, 1998; Schröder *et al.*, 1999; Agell *et al.*, 2001; Efremova *et al.*, 2002; Cebrian *et al.*, 2006; Schill *et al.*, 2006; López-Legentil *et al.*, 2008).

Several studies have been conducted to assess the effects of water-based drill cuttings/sedimentation to benthic communities (Trannum *et al.*, 2010; Trefry *et al.*, 2013). Offshore drilling activities discharge large amounts of waste material in the form of drill cuttings that cause increased sedimentation around oil and gas installations, which cause a significant reduction in the number of taxa, abundance, biomass and diversity of macrofauna in benthic communities (Trannum *et al.*, 2010). Water-based mud causes sedimentation and therefore physical stress to sessile benthic animals up to a distance of 500 m from the actual drilling site (Bakke *et al.*, 2013).

The marine sponge *Thenea muricata* (Bowerbank, 1858) (Demospongiae, Astrophorida, Theneidae, Thenea) has adapted to life in the deep sea (60–3000 m), preferably on soft muddy bottoms. The habitats of *T. muricata*, the most abundant species of the genus *Thenea*, are widely distributed in the Atlantic Ocean from the waters of North Greenland to the southern Mediterranean Sea (Cárdenas & Rapp, 2012). The data about molecular biology and biochemistry of this widely distributed soft bottom sponge are scarce: in one study the metabolic activity and oxygen consumption of *T. muricata* was determined (Witte & Graf, 1996), in another one the nucleotide profile of *T. muricata* was studied, which revealed the presence of unusual 2',5'-linked oligonucleotides, the natural metabolites of 2',5'-oligoadenylate synthetase (Lopp *et al.*, 2010). Cárdenas *et al.* (2011) sequenced a fragment of the mitochondrial cytochrome c oxidase I gene and

C1-D2 domain of 28S ribosomal gene, which are so far the only genetic data about *T. muricata* available in the GenBank.

In the framework of the SPONGRAM (Sponge Risk Assessment and Monitoring) project (2008–2009), whose main goal was to assess the potential negative impact on deep water sponge communities caused by elevated sedimentation rates associated with offshore oil and gas drilling activity, *T. muricata* was chosen to represent deep water sponges. The SPONGRAM project combined the background data of the sponge physiology under *in situ* conditions and laboratory data to assess cultivation and sedimentation effects on sponge survival, cell physiology and microbial community. The results of the project which suggested stress conditions of the sponges inspired us to study HSP70 expression in different sponge samples to gain more specific information about their stress response.

The aim of the present work was to study the expression of HSP70 during cultivation of *T. muricata* under laboratory conditions and subsequent sediment exposure experiments. Stress response of sponges was assessed on protein and mRNA levels. Several gene fragments from *T. muricata* coding for HSP70 family proteins were identified and the observed HSP70 set in the studied sponge samples was described.

MATERIALS AND METHODS

Sponge samples

Sponges were collected from the North Sea on a soft bottom sediment off Korsfjord (close to Bergen) at 300 m depths (60°07'44N 004°49'9E) in the framework of the SPONGRAM project (2008–2009). Sponges were shock frozen in liquid nitrogen directly on board the research vessel or transported to the laboratory for cultivation studies. The sponge samples were transported to the aquarium facility of the University of Bergen in water from the sampling site and maintained in natural running seawater from a depth of 200 m. They were cultivated in tanks with running seawater (seawater culture) or planted in sediment (sediment culture) and flushed continuously with running seawater (the temperature was 9–11°C in the cultivation tanks throughout the year). In sediment exposure experiments, sponges were continuously exposed during a 4-week period to a certain amount of sediment slurry dripping into the cultivation tank. One month after the start of cultivation, the sponges were exposed to 5 mg L⁻¹ of sediments, then the sediment addition was stopped for 4 weeks and the sponges were exposed to an increased load of sediments (10 mg L⁻¹). In two different experiments with the sediment loads of 10 and 30 mg L⁻¹, the addition to sponges started after 2 months of their maintaining in culture. Samples for analyses throughout the experimental period were shock frozen in liquid nitrogen and subsequently stored at -80°C. Every sample corresponded to a different sponge specimen.

From 30 samples of *T. muricata* chosen for the analysis, four were frozen *in situ* and 26 were cultivated in seawater or sediment tanks for different time periods. The number of cultivated sponges analysed at the beginning of cultivation studies (5 days) was five (three of them were dead), after 1 month of cultivation – four, after 2 months – six, after

3 months – six, after 4 months – four, and after 7 months of cultivation – one sponge.

Sponge protein extracts, SDS PAGE and Western blotting

Sponges were powdered at liquid nitrogen temperature by using tissue homogenizer TissueLyser (Qiagen, Retsch GmbH, Germany) at 30 Hz for 1 min. 50 µL of lysis buffer (140 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM Tris-HCl, pH 7.5) was added to 50–100 mg of a powdered sponge sample. The mixture was vortexed and kept on ice for 20 min followed by centrifugation (13,200 rpm, 4°C) for 15 min. The concentration of proteins remaining in the supernatant was determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) for calibration curve.

Proteins (20 µg of total protein) were separated using electrophoresis in 10% sodium dodecyl sulphate polyacrylamide gel (SDS PAGE) (Laemmli, 1970). Proteins were visualized by staining with PageBlue™ Protein Staining Solution (Thermo Fisher Scientific, Waltham, MA). For Western blot analysis proteins were blotted to Hybond C Extra membrane (Amersham®, GE Healthcare, Little Chalfont, UK) overnight at 175 mA. Membranes were blocked with 5% powdered skim milk in TBS-Tween 20 (140 mM NaCl, 0.1% Tween 20, 20 mM Tris-HCl, pH 7.5). Monoclonal Anti-Heat shock protein 70 (HSP70) clone BRM-22 (Sigma Aldrich, St. Louis, MO) diluted 1:5000 in TBS-Tween 20 was used as a primary antibody and goat anti-mouse IgG, F(ab')₂-HRP (Santa Cruz Biotechnology, Dallas, TX) in 1:5000 dilution was used as a secondary antibody. Western blot results were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce®, Thermo Fisher Scientific, Rockford, IL) and ImageQuant™ Las4000 equipment with attached software (GE Healthcare, Little Chalfont, UK).

RNA extraction and RT-PCR

Sponge RNA was extracted using TRIzol reagent (Invitrogen). 1 µg of RNA and 100 pmol of 3'-RACE adapter primer from FirstChoice® RLM-RACE kit (Ambion®, Thermo Fisher Scientific, USA) were used for cDNA synthesis with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). HotStarTaq® Plus PCR mixture (Qiagen, Hilden, Germany) with TmHSP70deg_F and TmHSP70deg_R primers (Table 1) were used for the first amplification of *T. muricata* HSP70 fragments from cDNA. The second PCR was conducted using the amplicon together with primers pJET_F and pJET_R* (Table 1). The first PCR profile was as follows: initial denaturation at 94°C for 5 min, followed by 10 cycles at 94°C for 1 min, 48°C for 1 min, 72°C for 3 min, followed by 25 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 3 min with final extension at 72°C for 10 min. The second PCR was conducted using the following profile: initial denaturation at 94°C for 5 min, followed by 25 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 3 min with final extension at 72°C for 10 min.

The PCR products were analysed in 1% agarose gel and DNA was visualized with ethidium bromide. The PCR products of an expected length (1500 bp) were purified from

Table 1. Primers used for amplification reactions and sequencing

Primer name	Primer sequence 5'→3'
TmHSP70 deg_F	CGACTCACTATAGGGAGACGGCGG(C/G/T) AC(A/C/G/T)AC(C/G/T)TA(C/T)TC(A/C/G/T) TG(C/T)GT
TmHSP70deg_R	AAGAACATCGATTTTTGGCAGCC(C/T)TT(A/G) TC(A/G)TT(A/C/G/T)GT(A/G/T)AT(A/G/T)GT
pJET_F	CGACTCACTATAGGGAGACGGC
pJET_R*	AAGAACATCGATTTTTGGCAG
TmC7GRP78_F	GGGAAGTTTGACCTCCAGGG
TmT2HSP70a_F	GGTATCCTCAACGTGTCTGC
TmC7/ C2.2HSP70a_F	GTGCCGAGATTGAAGTGAC
TmHSP70u_F	CAAGGGCGAGGAGAAGAC

*Modified pJET_R.

the gel using GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Purified PCR products were subjected to the direct sequencing from both ends using pJET_F and pJET_R* primers (Table 1) and subcloned using InsTAclone™ PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA). Plasmid DNA was extracted by alkaline lysis (Sambrook & Russell, 2001), the presence of an insert of an expected length was determined by restriction analysis. 3–5 plasmids with inserts of expected length were chosen for sequencing with pJET_F and pJET_R* primers (Table 1).

The 3'-RACE procedures were performed using FirstChoice® RLM-RACE kit (Ambion®, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. To obtain 3' regions of HSP70a coding mRNAs, the primer TmHSP70u_F (Table 1) was used in combination with the FirstChoice® RLM-RACE kit 3'-adaptor specific outer primer. Subsequently, the nested PCR was conducted with a 3'-adaptor specific inner primer in combination either with the primer TmT2HSP70a_F (designed on the basis of the obtained HSP70a sequence from the specimen T2) or the primer TmC7/C2.2HSP70a_F (designed on the basis of the obtained HSP70a sequences from the specimens C2.2 and C7, Table 1). Primer TmC7GRP78_F was used in combination with FirstChoice® RLM-RACE kit (Ambion®, Thermo Fisher Scientific, Waltham, MA) 3'-adaptor specific outer primer to obtain 3' regions of GRP78 mRNA from all three specimens analysed. PCR products were analysed in 1% agarose gel and DNA was visualized with ethidium bromide. PCR products of expected length (550–650 bp) were purified from the gel using GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems®, LifeTechnologies®, Thermo Fisher Scientific, Waltham, MA), and Genetic Analyzer 3130 (Applied Biosystems®, LifeTechnologies®, Thermo Fisher Scientific, Waltham, MA) was used for the analysis. Sequence manipulations and illustrations were made using the BioEdit program. The phylogenetic tree was constructed using online software: <http://www.phylogeny.fr> (Dereeper *et al.*, 2008).

Sequences of human, bacterial and known sponge HSP70 proteins used for comparison were obtained from the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>). GenBank accession

numbers of the sequences are indicated in the Supplementary Material, Appendix 3.

RESULTS

Immunological detection of *T. muricata* HSP70s

The representative set of the analysed samples is given in Figure 1. 70 kDa proteins were not detected in the sponges frozen directly on the research vessel (Figure 1, lanes T). At the beginning of cultivation and after 1 or 2 months cultivation in seawater or sediment tanks, low levels of HSP70 were detected in some, but not in all sponge samples (Figure 1, lanes C₁–C₂). Also, HSP70 was not detected in those sponges that died at the beginning of cultivation (Figure 1, lanes D). After longer cultivation (3 or more months), HSP70 was detected in every sample with generally higher expression levels (Figure 1, lanes C₃–C₇). In addition to the cultivation duration that had an obvious effect on HSP70 expression level, the latter was influenced by differences between sponge individuals subjected to the same treatments (see Figure 1, lanes C_{1.1} and C_{1.3}; C_{4.1}* and C_{4.2}*). However, the impact on HSP70 expression caused by elevated sediment load did not become evident from the sediment exposure experiments, e.g. see Figure 1 lanes C_{3.1}, C_{3.3}* and C_{3.5}*, which corresponded to sponge specimens cultivated without sediments, exposed to 5 or 30 mg L⁻¹ of sediments, respectively.

Remarkably, in all samples tested (except those that originated from two out of the three dead sponges, see Figure 1, lane D₁; D₃ not shown) cross-reactivity of bovine HSP70 antibody with proteins (or protein doublets) of about 60 kDa was observed. In some samples, proteins of about 100 and 50 kDa were also recognized. The identity of these proteins is not clear at present.

HSP70 mRNAs and conserved sequence motifs in *T. muricata*

To elucidate the genes of the HSP70 family proteins, three sponge specimens were chosen for a further analysis of HSP70 expression at mRNA level: specimens T₂, C_{2.2} and C₇. Specimen T₂ was a representative of samples frozen on the research vessel, C_{2.2} represented short-term cultivated samples, and C₇ – long-term cultivated samples.

Initially, 1500 bp fragments of HSP70 cDNA were amplified using degenerate primers that were designed on the basis of previously known HSP70 sequences of sponges and other animals. The obtained fragments included the majority of the N-terminal nucleotide (ATP) binding domain (NBD) and most of the substrate (peptide) binding domain (SBD) of HSP70s (Figure 2) – thus covering the most conserved part of HSP70s. Subcloning and sequencing revealed the presence of three types of proteins belonging to the HSP70 superfamily: HSP70a type sequences were detectable in all three specimens, GRP78 was detectable in the cultivated specimens C_{2.2} and C₇, HSP70b was present only in the specimen T₂ (frozen *in situ*). Two HSP70a sequences that were obtained from the specimen T₂ had a few single nucleotide differences, but none of those led to amino acid substitution. In addition, one HSP70b type sequence was identified from T₂, while GRP78 was not detectable in this specimen. Three HSP70a sequences sharing a 99% sequence identity on amino acid level were obtained for C_{2.2}; GRP78 sequence was detectable on the electropherogram of direct sequencing as minor peaks under major peaks of HSP70a sequence. On the contrary, GRP78 was obtained as the main sequence from the long-term cultivated specimen C₇; the HSP70a sequence was detected under major peaks of GRP78 sequence. According to blastp search in non-redundant protein sequences database of NCBI, HSP70a type had best matches with stress-induced or constitutive HSP70 proteins from sponges and other organisms. The GRP78 from *T. muricata* had best matches with another member of the HSP70 superfamily – glucose-regulated protein of 78 kDa. HSP70b type protein sequence had best BLAST matches mainly with amoeboid HSP70 sequences, indicating that it might originate from a sponge-associated eukaryotic microorganism.

As the primer HSP70deg_F was designed on the basis of signature sequence 1, specific to all HSP70 proteins, only the other two HSP70 signatures defined in PROSITE database of protein domains (Bairoch, 1992) are present in HSP70 sequences of *T. muricata*: signature 2 DLGGGFD, identical in all sequences (positions 184–191, Supplementary Material, Appendix 1), and signature 3 [LI]VLVGGSTRIPK[IV]Q[KE] having slight differences between HSP70a, HSP70b and GRP78 (positions 319–333, Supplementary Material, Appendix 1). The additional conserved motifs Cons1–Cons3, defined by Rensing & Maier (1994), are also detectable in all obtained sequences, including conserved motif 3 characteristic of eukaryotic non-mitochondrial or non-plastid HSP70s (Figure 2; Supplementary Material, Appendix 1).

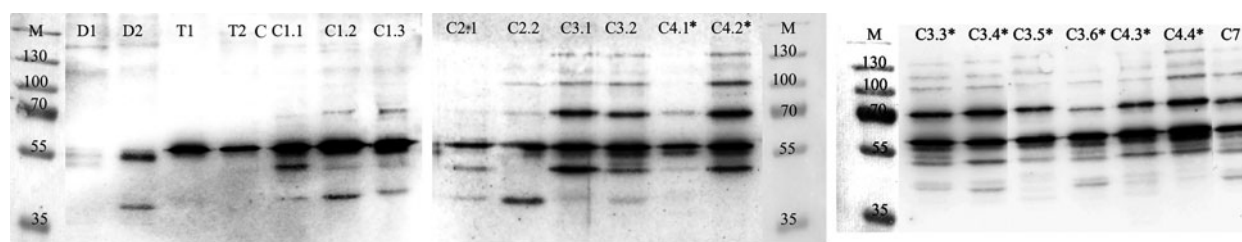


Fig. 1. The proteins recognized by bovine HSP70 antibody in the samples of *T. muricata*. Names above the lanes indicate particular specimens. Symbols in the names are as follows: D, specimens that were dead; T, specimens that were frozen on research vessel; C, cultivated specimens: the first number indicates cultivation time in months and the second number indicates the particular specimen. *, specimens exposed to sediments, in particular C_{3.3}* and C_{3.4}* correspond to 5 mg L⁻¹; C_{3.5}* and C_{3.6}* correspond to 30 mg L⁻¹; C_{4.1}* and C_{4.2}* correspond to 5 mg L⁻¹ + 10 mg L⁻¹; C_{4.3}* and C_{4.4}* correspond to 10 mg L⁻¹ of sediment load. Lane M – molecular weight marker (kDa).

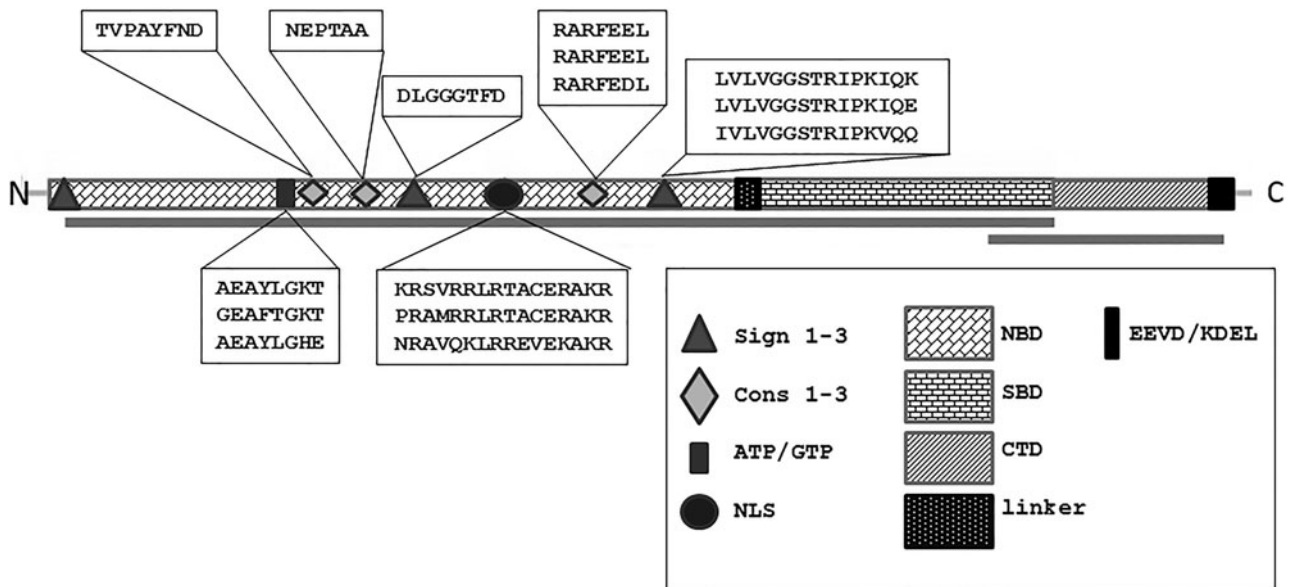


Fig. 2. The topology and conserved motifs of HSP70 proteins from *T. muricata*. Three domains of HSP70 superfamily proteins are shown: the nucleotide binding domain (NBD), the substrate binding domain (SBD) and the C-terminal domain (CTD). HSP70 specific signature sequences (Sign 1–3), conserved motifs (Cons 1–3), the ATP/GTP binding sequence and the nuclear localization signal (NLS) are located in NBD. Proteins of HSP70 superfamily end with the EEVD/KDEL motif specific for HSP70/GRP78, respectively. The regions amplified from *T. muricata* are indicated as lines below the topology chart. The sequences of the motifs found in *T. muricata* HSP70 proteins are written in textboxes (if the motif sequences are not identical they belong to HSP70a, HSP70b or GRP78 proteins, respectively).

A potential ATP/GTP binding P-loop motif [AG]x(4)GK[ST] (Saraste *et al.*, 1990) is present in all sequences (positions 116–123) with minor differences in C7_GRP78.

Nuclear localization signal NLS (positions 241–257, Supplementary Material, Appendix 1) is present in HSP70a sequences but in GRP78 and HSP70b the first position of the signal has been substituted, suggesting that those proteins do not locate to nucleus. Several specific single amino acid positions have been identified in literature that distinguish between eukaryotic and prokaryotic, ER and cytoplasmic HSP70s (Gupta *et al.*, 1994). Positions that can be used to distinguish between cytosolic and ER HSP70s are highlighted in the Supplementary Material, Appendix 1; those positions support the specification of *T. muricata* HSP70 sequences.

3'-RACE was performed to amplify 3' regions of obtained HSP70 mRNAs to further confirm the specification of the obtained sequences. The eukaryotic HSP70s that are located in cytoplasm usually end with regulatory sequence EEVD whereas GRP78 proteins end with ER retention signal KDEL (Munro & Pelham, 1987). Our 3'-RACE results for HSP70a of the specimen C2.2, for GRP78 of the specimen C7 and for HSP70a of the specimen T2 supported the previous results. Additionally, using primers designed on the basis of GRP78 sequence from C7, a 3' region was obtained for GRP78 of the specimen C2.2. An additional 3' region that ends with EEVD signature was obtained for C2.2, which was not compatible with the previously known sequences (Supplementary Material, Appendix 2). No 3'-RACE result was obtained for HSP70b, so the true identity of this protein remains obscure.

The obtained sequences of *T. muricata* which were deposited to Genbank have the following accession numbers: KP863863 (GRP78_spC7), KP863864 (HSP70a_spC7), KP863865 (HSP70a1_spT2), KP863866 (HSP70a2_spT2), KP863867

(HSP70a1_spC2.2), KP863868 (HSP70a2_spC2.2), KP863869 (HSP70a3_spC2.2) and KP863870 (HSP70b_spT2).

Multiple sequence alignment and phylogenetic analysis

T. muricata HSP70 sequences were compared with HSP70 sequences from other sponge species and from human as well as with bacterial DnaK protein sequence. Only full length sequences of sponge HSP70s were taken for comparison. From the human HSP70 protein sequences HSPA1A was selected as a representative of cytosolic stress-induced HSP70s, HSPA8 as a representative of constitutively expressed cytosolic HSP70s and HSPA5 as a representative of ER located GRP78s.

The C-terminal region of HSP70s is weakly conserved (Rensing & Maier, 1994), thus only the sequences obtained from the degenerate PCR experiment from *T. muricata* and the corresponding regions of the sequences from other organisms (including the conserved region of HSP70s) were used for the multiple alignment. An identity/similarity matrix (Supplementary Material, Appendix 4) was constructed on the basis of multiple sequence alignment of the above mentioned HSP70 family protein sequences. HSP70a type sequences from *T. muricata* had sequence identities and similarities with stress-induced or constitutive HSP70s in the range of 75–90% and 86–95%, respectively; identities and similarities with GRP78 protein sequences were lower. The GRP78 sequence of *T. muricata* (the specimen C7) was more similar to glucose-regulated proteins from sponges and humans, whereas the identities and similarities with HSP70s were lower. The HSP70b sequence was more similar to the HSP70 than the GRP78 protein sequences (Supplementary Material, Appendix 4). A phylogenetic tree

was constructed on the basis of known sponge HSP70 protein sequences. All HSP70 proteins of *T. muricata* branch together with the HSP70 superfamily proteins from other sponges. GRP78 is more closely related to GRP78 from other sponges while HSP70a is grouped together with the stress-induced or constitutive HSP70s (Figure 3).

DISCUSSION

In the course of the SPONGRAM project, cultures of *T. muricata* were successfully established for *ex situ* impact studies that enabled us to assess the cultivation effect as well as the impact of elevated sediment load on sponge survival and cell physiology. There was a high mortality of sponges at the beginning of cultivation, however, after an adaptation period of 1–2 months the populations became stable. Also, the metabolic activity estimated by adenylate energy charge (AEC) values indicated a beneficial effect of long-term cultivation (4–7 months) of sponges: AEC values increased after 3 months of cultivation (results published in the final report of the SPONGRAM project). However, the effect of long-term cultivation on the expression of proteins of the HSP70 family in sponges had not been studied before our research project. Long-term cultivation of sponges in laboratory conditions, despite many studies that have been devoted to the subject, is still a difficult task with many questions yet to be answered (Schippers *et al.*, 2012).

The results presented in this work indicate that 70 kDa proteins were detected immunologically at different levels in *T. muricata* samples. In preliminary experiments, two HSP70 antibodies (polyclonal *Geodia cydonium* HSP70 and monoclonal bovine brain HSP70) were tested for their suitability to recognize 70 kDa proteins in different samples. Interestingly, the marine sponge antibody produced against recombinant HSP70 from *G. cydonium* was not able to detect 70 kDa proteins in the samples of *T. muricata* from the adaptation period (1–2 months) of cultivated sponges (data not shown). The proteins of 70 kDa from those samples were detected at low levels using bovine HSP70

antibody (Figure 1); therefore, the latter antibody was used for the subsequent immunological studies. Higher levels of the stress protein HSP70 observed after 3 months of cultivation seemed to be in conflict with the adaptation of sponges to culture conditions and the improved physiological status of sponges (as assessed by AEC values (Witte & Graf, 1996)) after the adaptation period. However, these data may suggest that though the mortality of sponges had been reduced to a minimum, the maintenance of the remaining sponges in culture needs a continuous induction of HSP70 proteins. As there was no clear effect of elevated sediment load on the levels of HSP70, possible sedimentation effects (if they were manifested at all) may have been masked by the cultivation effects or by the variations among different sponge individuals. Some tolerance to sedimentation effects may be characteristic of this sponge species, as *T. muricata* lives on soft muddy bottoms and, interestingly, as revealed from sediment exposure experiments, the sponges can use their basal spicules to ‘crawl’ out of the sediment (H.T. Rapp, unpublished data).

In addition to 70 kDa proteins, bovine HSP70 antibody recognized several other proteins in the extracts of *T. muricata*: primarily the proteins of about 60 kDa were detected in the cultivated samples as well as in those frozen *in situ* (Figure 1). Similarly, bovine HSP70 antibody recognized the proteins of about 60 kDa in the case of marine sponges *Crambe crambe* and *Chondrosia reniformis* (Agell *et al.*, 2001; Cebrian *et al.*, 2006). Agell *et al.* (2001) explained this phenomenon as a cross-reaction of the specific antibody with the proteins from two different HSP (HSP60 and HSP70) families. Yet another possibility may exist according to which the protein(s) of about 60 kDa is (are) splice variant(s) of a member of the proteins belonging to the HSP70 family. Tsukahara *et al.* (2000) have characterized HSC54, a novel 54 kDa splice-variant of the human heat-shock cognate protein 70. The true nature/origin of the proteins of around 60 kDa as well as that of the other proteins recognized by bovine HSP70 antibody in *T. muricata* remains to be elucidated in future studies.

The specification of particular 70 kDa proteins (stress-induced, constitutive, GRP) recognized by bovine HSP70 by means of the Western blot analysis is hardly possible. Both identification by mass-spectrometry and quantification by qPCR that would allow advanced measurements of HSP70 expression levels, require pre-known sequence data that have not been available for *T. muricata* so far. So our next task was to identify which mRNAs coding for HSP70 proteins could be detected in *T. muricata* samples. For that three specimens were chosen with different levels of immunologically detectable 70 kDa proteins. We identified three types of HSP70 coding mRNAs in *T. muricata*; at that, different sets of HSP70 mRNAs were obtained for different specimens. Sequence motifs indicate that the proteins coded by those mRNAs are either stress-induced or constitutively expressed members of HSP70 and GRP78 families. One obtained sequence – HSP70b – seems to be a 70 kDa stress protein from a sponge-associated symbiotic organism. Such a result is not surprising since sponges are known to harbour large amounts of bacterial and eukaryotic symbionts (Fan *et al.*, 2012). The finding of different sets of mRNAs coding for proteins of HSP70 family gives the first implication of the distribution of the corresponding proteins in the specimens of *T. muricata*, which were subjected to different

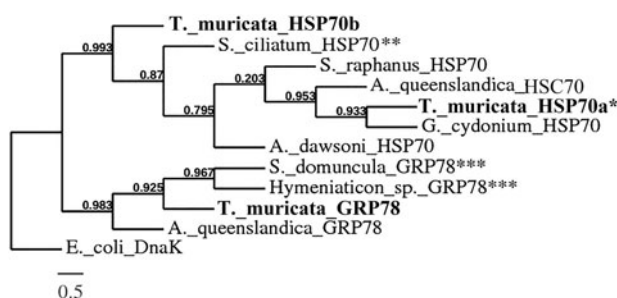


Fig. 3. A phylogenetic tree based on multiple sequence alignment of amino acid sequences of the HSP70 superfamily proteins from sponges (Supplementary Material, Appendix 3). Branch support values are indicated on branches (the branch length is proportional to the number of substitutions per site). The sequences obtained from *T. muricata* are shown in bold. *HSP70a1 from specimen C2.2 was chosen for the phylogenetic analysis as a representative of *T. muricata* HSP70a type sequences. **The protein has been deposited to the database as GRP78, but due to the EEVD motif at the end of the sequence, the protein was renamed as HSP70. ***The protein has been deposited to the database as HSP70, but due to the ER retention signal KDEL at the end of the sequence, the protein was renamed as GRP78.

treatments. The data obtained from direct sequencing experiments show that for the specimen T2 (frozen *in situ*, no immunologically detectable HSP70), the obtained sequence corresponded to HSP70a. For the specimen C2.2 (short-term cultivation, HSP70 detected at low levels), the main sequence corresponded to HSP70a; in addition, GRP78 was identified on the basis of accompanying minor peaks. For the specimen C7 (long-term cultivation, HSP70 detected at higher levels), the main sequence corresponded to GRP78; HSP70a was identified from accompanying minor peaks. Although direct sequencing is not as good as the qPCR method for quantification purposes, it still gives us a hint about the distribution of different HSP70 mRNAs (and the corresponding proteins) in the particular sponges. These data allow us to speculate that the induction of GRP78, which may be the result of the accumulation of misfolded proteins inside the ER, is important for long-term cultivation of *T. muricata*. However, high levels of any of the stress proteins point to persistent stress conditions of sponges that survived the adaptation period and had been maintained for months under laboratory conditions.

In addition, the repertoire of HSP70s in sponges, including *T. muricata*, may be much wider than presently known. Six mRNAs sequences from different sponge species, covering the complete coding part of HSP70s, have been deposited to GenBank: four of them were cytosolic stress-induced HSP70s and two belonged to the GRP78 family. Furthermore, a number of mRNA sequences that code for proteins of the HSP70 superfamily can be found in *Amphimedon queenslandica* (Conaco *et al.*, 2012), the only sponge with a published genome (Srivastava *et al.*, 2010). At least 17 genes coding for proteins belonging to the HSP70 superfamily have been described in humans (Brocchieri *et al.*, 2008). Besides, considering that proteins from several different HSP superfamilies may contribute to the stress response of sponges under various conditions, the detailed understanding of the contribution of each stress protein involved calls for future studies.

In conclusion, the results of the present study indicate that HSP70 expression levels in *T. muricata* were mainly affected by the duration of cultivation of sponges. After a cultivation period of 2–3 months when sponges had adapted to life in culture, persistent higher levels of HSP70 expression were observed. Our data referred to changes of the pattern of induced stress proteins during long-term cultivation of sponges. The involvement of ER stress protein GRP78 was evidently needed to provide supplementary protection for the survival of cultivated sponges. The results obtained in this study provide a basis for future quantitative stress studies in this field.

FINANCIAL SUPPORT

This work was supported through the SPONGRAM project funded by The Norwegian Deep Sea Program (NDP) through contract number 4501533230, and also by the Estonian Ministry of Education and Research (grant No. 0140108) and the Estonian Science Foundation (grant No. 9185).

Supplementary materials

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0025315415002234>

REFERENCES

- Agell G., Uriz M.J., Cebrian E. and Martí R. (2001) Does stress protein induction by copper modify natural toxicity in sponges? *Environmental Toxicology and Chemistry* 20, 2588–2593.
- Bairoch A. (1992) PROSITE: a dictionary of sites and patterns in proteins. *Nucleic Acids Research* 20, 2013–2018.
- Bakke T., Klungsoyr J. and Sanni S. (2013) Environmental impacts of produced water and drilling waste discharges from the Norwegian offshore petroleum industry. *Marine Environmental Research* 92, 154–169.
- Bardwell J.C. and Craig E.A. (1984) Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible *dnaK* gene are homologous. *Proceedings of the National Academy of Sciences USA* 81, 848–852.
- Batel R., Bihari N., Rinkevich B., Dapper J., Schacke H., Schroder H.C. and Müller W.E.G. (1993) Modulation of organotin-induced apoptosis by the water pollutant methyl mercury in a human lymphoblastoid tumor cell line and a marine sponge. *Marine Ecology Progress Series* 93, 245–251.
- Borchiellini C., Boury-Esnault N., Vacelet J. and Le Parco Y. (1998) Phylogenetic analysis of the Hsp70 sequences reveals the monophyly of Metazoa and specific phylogenetic relationships between animals and fungi. *Molecular Biology and Evolution* 15, 647–655.
- Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254.
- Brocchieri L., Conway de Macario E. and Macario A.J.L. (2008) hsp70 genes in the human genome: conservation and differentiation patterns predict a wide array of overlapping and specialized functions. *BMC Evolutionary Biology* 8, 19.
- Buchberger A., Bukau B. and Sommer T. (2010) Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. *Molecular Cell* 40, 238–252.
- Cárdenas P. and Rapp H.T. (2012) A review of Norwegian streptaster-bearing Astrophorida (Porifera: Demospongiae: Tetractinellida), new records and a new species. *Zootaxa* 3253, 1–53.
- Cárdenas P., Xavier J.R., Reveillaud J., Schander C. and Rapp H.T. (2011) Molecular phylogeny of the Astrophorida (Porifera, Demospongiae(p)) reveals an unexpected high level of spicule homoplasmy. *PLoS ONE* 6, e18318.
- Cebrian E., Agell G., Martí R. and Uriz M.J. (2006) Response of the Mediterranean sponge *Chondrosia reniformis* Nardo to copper pollution. *Environmental Pollution* 141, 452–458.
- Conaco C., Neveu P., Zhou H., Arcila M.L., Degnan S.M., Degnan B.M. and Kosik K.S. (2012) Transcriptome profiling of the demosponge *Amphimedon queenslandica* reveals genome-wide events that accompany major life cycle transitions. *BMC Genomics* 13, 209.
- Dereeper A., Guignon V., Blanc G., Audic S., Buffet S., Chevenet F., Dufayard J.F., Guindon S., Lefort V., Lescot M., Claverie J.M. and Gascuel O. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* 36, W465–W469.
- Efremova S.M., Margulis B.A., Guzhoval I.V., Itskovich V.B., Lauenroth S., Müller W.E.G. and Schröder H.C. (2002) Heat shock protein Hsp70 expression and DNA damage in Baikalian sponges exposed to model pollutants and wastewater from Baikalsk Pulp and Paper Plant. *Aquatic Toxicology* 57, 267–280.
- Fan L., Liu M., Simister R., Webster N.S. and Thomas T. (2013) Marine microbial symbiosis heats up: the phylogenetic and functional response of a sponge holobiont to thermal stress. *ISME Journal* 7, 991–1002.

- Fan L., Reynolds D., Liu M., Stark M., Kjelleberg S., Webster N. and Thomas T. (2012) Functional equivalence and evolutionary convergence in complex communities of microbial sponge symbionts. *Proceedings of the National Academy of Sciences USA* 109, E1878–1887.
- Feder M.E. and Hofmann G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* 61, 243–282.
- Filicetto F., Vazzana M., Celi M., Maccarrone V., Ceraulo V.M., Buffa C., Di Stefano V., Mazzola S. and Buscaino G. (2014) Behavioural and biochemical stress responses of *Palinurus elephas* after exposure to boat noise pollution in tank. *Marine Pollution Bulletin* 84, 104–114.
- Gupta R.S., Aitken K., Falah M. and Singh B. (1994) Cloning of *Giardia lamblia* heat shock protein HSP70 homologs: implications regarding origin of eukaryotic cells and of endoplasmic reticulum. *Proceedings of the National Academy of Sciences USA* 91, 2895–2899.
- Hendrick J.P. and Hartl F. (1993) Molecular chaperone functions of heat-shock proteins. *Annual Review of Biochemistry* 62, 349–384.
- Kozioł C., Kobayashi N., Müller I.M. and Müller W.E.G. (1998) Cloning of sponge heat shock proteins: evolutionary relationships between the major kingdoms. *Journal of Zoological Systematics and Evolutionary Research* 36, 101–109.
- Kozioł C., Leys S.P., Müller I.M. and Müller W.E.G. (1997) Cloning of Hsp70 genes from the marine sponges *Sycon raphanus* (Calcarea) and *Rhabdocalypus dawsoni* (Hexactinellida). An approach to solve the phylogeny of sponges. *Biological Journal of the Linnean Society* 62, 581–592.
- Kozioł C., Wagner-Hülsmann C., Mikó A., Gamulin V., Kruse M., Pancer Z., Schäcke H. and Müller W.E.G. (1996) Cloning of a heat-inducible biomarker, the cDNA encoding the 70 kDa heat shock protein, from the marine sponge *Geodia cydonium*: response to natural stressors. *Marine Ecology Progress Series* 136, 153–161.
- Kregel K.C. (2002) Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *Journal of Applied Physiology* 92, 2177–2186.
- Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lafferty K.D., Porter J.W. and Ford S.E. (2004) Are diseases increasing in the ocean? *Annual Reviews in Ecology, Evolution and Systematics* 35, 31–54.
- Lee A.S. (2014) Glucose-regulated proteins in cancer: molecular mechanisms and therapeutic potential. *Nature Reviews Cancer* 14, 263–276.
- López-Legentil S., Song B., McMurray S.E. and Pawlik J.R. (2008) Bleaching and stress in coral reef ecosystems: hsp70 expression by the giant barrel sponge *Xestospongia muta*. *Molecular Ecology* 17, 1840–1849.
- Lopp A., Reintamm T., Kuuskalu A., Tammiste I., Pihlak A. and Kelve M. (2010) Natural occurrence of 2',5'-linked heteronucleotides in marine sponges. *Marine Drugs* 8, 235–254.
- Mayer M.P. and Bukau B. (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences* 62, 670–684.
- Müller W.E.G., Batel R., Lacorn M., Steinhart H., Simat T., Lauenroth S., Hassanein H. and Schröder H.C. (1998) Accumulation of cadmium and zinc in the marine sponge *Suberites domuncula* and its potential consequences on single-strand breaks and on expression of heat-shock protein: a natural field study. *Marine Ecology Progress Series* 167, 127–135.
- Müller W.E.G., Kozioł C., Dapper J., Kurelec B., Batel R. and Rinkevich B. (1995) Combinatory effects of temperature stress and nonionic organic pollutants on stress protein (hsp70) gene expression in the freshwater sponge *Ephydatia fluviatilis*. *Environmental Toxicology and Chemistry* 14, 1203–1208.
- Munro S. and Pelham H.R.B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48, 899–907.
- Rensing S.A. and Maier U.G. (1994) Phylogenetic analysis of the stress-70 protein family. *Journal of Molecular Evolution* 39, 80–86.
- Rossi S. and Snyder M.J. (2001) Competition for space among sessile marine invertebrates: changes in HSP70 expression in two Pacific cnidarians. *Biological Bulletin* 201, 385–393.
- Sambrook J. and Russell D.W. (2001) Preparation of plasmid DNA by alkaline lysis with SDS: miniprep. In Argentine J., Irwin N. et al. (eds) *Molecular cloning: a laboratory manual*. Volume 1. 3rd edition. New York, NY: Cold Spring Harbor Laboratory Press, pp. 1.32–1.34.
- Sanders B.M. (1993) Stress proteins in aquatic organisms: an environmental perspective. *Critical Reviews in Toxicology* 23, 49–75.
- Saraste M., Sibbald P.R. and Wittinghofer A. (1990) The P-loop – a common motif in ATP- and GTP-binding proteins. *Trends in Biochemical Sciences* 15, 430–434.
- Schill R.O., Pfannkuchen M., Fritz G., Köhler H.R. and Brümmer F. (2006) Quiescent gemmules of the freshwater sponge, *Spongilla lacustris* (Linnaeus, 1759), contain remarkably high levels of Hsp70 stress protein and hsp70 stress gene mRNA. *Journal of Experimental Zoology Part A: Comparative Experimental Biology* 305A, 449–457.
- Schippers K.J., Sipkema D., Osinga R., Smidt H., Pomponi S.A., Martens D.E. and Wijffels R.H. (2012) Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Advantages in Marine Biology* 62, 273–337.
- Schröder H.C., Batel R., Lauenroth S., Hassanein H.M.A., Lacorn M., Simat T., Steinhart H. and Müller W.E.G. (1999) Induction of DNA damage and expression of heat shock protein HSP70 by polychlorinated biphenyls in the marine sponge *Suberites domuncula* Olivi. *Journal of Experimental Marine Biology and Ecology* 233, 285–300.
- Srivastava M., Simakov O., Chapman J., Fahey B., Gauthier M.E., Mitros T., Richards G.S., Conaco C., Dacre M., Hellsten U., Larroux C., Putnam N.H., Stanke M., Adamska M., Darling A., Degnan S.M., Oakley T.H., Plachetzki D.C., Zhai Y., Adamski M., Calcino A., Cummins S.F., Goodstein D.M., Harris C., Jackson D.J., Leys S.P., Shu S., Woodcroft B.J., Vervoort M., Kosik K.S., Manning G., Degnan B.M. and Rokhsar D.S. (2010) The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature* 466, 720–726.
- Trannum H.C., Nilsson H.C., Schaanning M.T. and Øxnevad S. (2010) Effects of sedimentation from water-based drill cuttings and natural sediment on benthic macrofaunal community structure and ecosystem processes. *Journal of Experimental Marine Biology and Ecology* 383, 111–121.
- Trefry J.H., Dunton K.H., Trocine R.P., Schonberg S.V., McTigue N.D., Hersh E.S. and McDonald T.J. (2013) Chemical and biological assessment of two offshore drilling sites in the Alaskan Arctic. *Marine Environmental Research* 86, 35–45.
- Tsukahara F., Yoshioka T. and Muraki T. (2000) Molecular and functional characterization of HSC54, a novel variant of human heat-shock cognate protein 70. *Molecular Pharmacology* 58, 1257–1263.
- Webster N., Pantile R., Botté E., Abdo D., Andreakis N. and Whalan S. (2013) A complex life cycle in a warming planet:

gene expression in thermally stressed sponges. *Molecular Ecology* 22, 1854–1868.

and

Witte U. and Graf G. (1996) Metabolism of deep-sea sponges in the Greenland-Norwegian Sea. *Journal of Experimental Marine Biology and Ecology* 198, 223–235.

Correspondence should be addressed to:

K. Vallmann

Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, Tallinn 12618, Estonia

email: kerli.vallmann@gmail.com