The coagulation system helps control *P. dicentrarchi* infection in the turbot *Scophthalmus maximus* (L.)

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Running title

The turbot coagulation system and *P. dicentrarchi*

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Abstract

Many studies have shown that coagulation systems play an important role in the defence against pathogens in invertebrates and vertebrates. In vertebrates, particularly in mammals, it has been established that the coagulation system participates in the entrapment of pathogens and activation of the early immune response. However, functional studies investigating the importance of the fish coagulation system in host defence against pathogens are scarce. In the present study, injection of turbot (*Scopthalamus maximus*) with the pathogenic ciliate *Philasterides dicentrarchi* led to the formation of macroscopic intraperitoneal clots in the fish. The clots contained abundant, immobilized ciliates, many of which were lysed. We demonstrated that the plasma clots immobilize and kill the ciliates *in vitro*. To test the importance of plasma clotting in ciliate killing, we inhibited the process by adding a tetrapeptide known to inhibit fibrinogen/thrombin clotting in mammals. Plasma tended to kill *P. dicentrarchi* slightly faster when clotting was inhibited by the tetrapeptide, although the total mortality of ciliates was similar. We also found that kaolin, a particulate activator of the intrinsic pathway in mammals, accelerates plasma clotting in turbot. In addition, PMA-stimulated neutrophils, living ciliates and several ciliate components such as cilia, proteases and DNA also displayed procoagulant activity *in vitro*. Injection of fish with the ciliates generated the massive release of neutrophils to the peritoneal cavity, with formation of large aggregates in those fish with live ciliates in the peritoneum. We observed, by SEM, numerous fibrin-like fibres in the peritoneal exudate, many of which were associated with peritoneal leukocytes and ciliates. Expression of the CD18/CD11b gene, an integrin associated with cell adhesion and the induction of fibrin formation, was upregulated in the peritoneal leukocytes. In conclusion, the findings of the present study show that *P. dicentrarchi* induces the formation of plasma clots and that the fish coagulation system may play an important role in immobilizing and killing this parasite.

Keywords: fish; *Philasterides dicentrarch*i; coagulation system; neutrophils; plasma; complement

1. Introduction

The coagulation system plays an important role in homeostasis and also in the defence against pathogens in invertebrates and vertebrates. The blood clots formed generate a competent barrier that prevents the spread of pathogens into the circulation (Sun, 2006). This system, which appeared early on in evolution and has been demonstrated in invertebrates such as the horseshoe crab (*Limulus polyphemus*) (Iwanaga and Kawabata, 1998), is considered one of the major defence systems in invertebrates (Iwanaga and Lee, 2005). The mammalian coagulation system consists of a cascade of enzyme activation events that culminate in the formation of a fibrin clot. The blood clotting cascade can be triggered by two major routes, known as the tissue factor pathway and the contact pathway (Smith et al., 2015). The former of these, also known as the the extrinsic pathway, functions in normal haemostasis and probably also in many types of thrombosis. The contact pathway, also known as the intrinsic pathway, is triggered when plasma comes into contact with certain types of surfaces (Smith et al., 2015). Tissue factor is the major initiator of the extrinsic pathway in mammals, which is mainly expressed by cell vessel walls and is released when the vessel wall is disrupted (Gaertner and Massberg, 2016). The contact system can be activated by non-physiological and physiological compounds such as kaolin, dextran sulphate, polyphosphate, neutrophil extracellular traps (NETs), nucleic acids and collagen, and also by pathogens such as bacteria and viruses (Nickel and Renné, 2012; Long et al., 2016). Interestingly, deficiencies in the contact pathway factors lead to prolonged clotting times *in vitro*, but do not cause bleeding complications *in vivo* (Maas and Renné, 2012).

During bacterial infections, the coagulation system cooperates with the immune system to eliminate the invading pathogens. Studies in the horseshoe crab have shown that the coagulin blood clot immobilizes microbes and acts together with plasma components to destroy clot-entrapped microbes (Isakova and Armstrong, 2003). In mammals, activation of the tissue factor pathway is considered part of the host defence to infection, and a protective role against certain pathogens has been described (van der Poll and Herwald, 2014). Many bacterial species have been shown to activate the contact pathway by different mechanisms (Nickel and Renné, 2012). The contact pathway acts synergistically by entrapping bacteria with fibrin and enhancing proinflammatory signalling, which suggests that coagulation is important in preventing

the spread of the invading pathogen (Frick et al., 2006; Loof et al., 2014; Nordahl et al., 2005).

Knowledge of the coagulation system in fish is rather limited. Although most coagulation factors found in mammals appear to occur in teleosts such as zebrafish (*Danio rerio*) and puffer fish (*Fugu rubripes*) (Davidson et al., 2003; Doolittle, 2015; Weyand and Shavit, 2014), some genes involved in the contact system, such as factor XII and prekallikrein, have not been found in fish (Doolittle, 2011). It has been suggested that the cascade equivalent to the plasma kallikrein-kinin system in mammals is absent in teleosts (Wong and Takei, 2013). Nonetheless, both the intrinsic and extrinsic coagulation pathways have been described in zebrafish (Jagadeeswaran and Sheehan, 1999). However, as far as we know, there is no information available about the role of the coagulation system in defence against pathogens in fish.

Philasterides dicentrarchi is an opportunistic histophagous ciliate that causes severe mortalities in cultured fish worldwide (Harikrishnan et al., 2010). Ciliates probably penetrate the fish through lesions in the gills or the skin and then proliferate in internal organs, causing systemic infection (Paramá et al., 2003). Fish infected experimentally with *P. dicentrarchi* show an intense inflammatory response that affects most organs (Puig et al., 2007) as well as upregulation of many genes involved in the immune response (Pardo et al., 2012). The ciliate and some of its components induce strong activation of turbot leukocytes; however, their role in defence against this pathogen seems to be minor, at least in comparison with humoral factors (Piazzon et al., 2011a, 2013). In this respect, fish complement appears to be a critical component in the defence against *P. dicentrarchi*, especially after activation of the classic pathway (Leiro et al., 2008; Piazzon et al., 2011a, 2013). In addition to complement, fish plasma also contains other soluble components, such as those forming part of the coagulation system, which may also have a role in controlling *P. dicentrarchi* infection. This study was undertaken to determine whether ciliates and their components can induce activation of the coagulation system and how coagulation affects the survival of *P. dicentrarchi*.

2. Materials and methods

2.1. Ciliates

Specimens of the ciliate *P. dicentrarchi* (isolate I1; Budiño et al., 2011) were aseptically isolated from ascites of naturally infected turbot. The ciliates were maintained at 18 °C in complete sterile L-15 medium (Leibovitz, Sigma-Aldrich, pH 7.2) containing adenosine, cytidine and uridine (90 mg/L), guanosine (150 mg/L), glucose (5 g/L), L-α-phosphatidylcholine (400 mg/L), Tween 80 (200 mg/L) and 10% heat inactivated foetal calf serum (Iglesias et al., 2003). The virulence of the ciliates was maintained by experimentally infecting samples of fish every 3 months by intraperitoneal injection, as previously described (Leiro et al., 2008). The ciliates were washed in L-15 medium by centrifugation (700 x g for 10 min) and resuspended in 3.5% NaCl, and the concentration was estimated with a haemocytometer. Alternatively, ciliates were resuspended in 3.5% NaCl containing 1 mM phenylmethylsufonyl fluoride (PMSF, dissolved in ethanol) and ultrasound was applied to the ciliate suspension on a bed of ice, in order to totally lyse the cells. Live and lysed ciliates were used in different clotting experiments.

To obtain the cilia, the ciliate suspension was centrifuged at 700 x g for 10 min, and the pellet was resuspended in L-15 medium containing 2.6 mM of dibucaine and incubated for 10 min (Thompson et al., 1974). Deciliation was monitored by observing the ciliate suspension under phase contrast microscopy. Cilia were separated from the other ciliate components by differential centrifugation, as indicated by Adoutte et al., (1980). The ciliate suspension was centrifuged at 700 x g for 10 min to eliminate other cell components derived from broken ciliates. The supernatant was centrifuged again at 15000 x g for 15 min and the pellet was resuspended in distilled water and stored at -80 ^{o}C. The protein content of the samples was determined by the Bradford assay, as indicated by Piazzon et al., (2008).

The *P. dicentrarchi* DNA was purified using the DNAesy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. The quality, purity and concentration of DNA were estimated by A_{260} measurement in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA.).

Ciliate proteases were purified on a bacitracin–Sepharose affinity column, as described by Piazzon et al., (2011b). Briefly, ciliates isolated from 0.5 L of *P. dicentrarchi* culture (about 10^5 ciliates/mL) were washed with PBS, resuspended in 5 mL of equilibration buffer (100 mM CH_3COONH_4, pH 6·5), sonicated on ice until broken and filtered (0.22 μm). The ciliate samples were then applied to a bacitracin–CNBr-activated sepharose 4B (GE Healthcare, Madrid, Spain) XK 16/20 column

connected to a protein purification system (ÄKTAprime™plus; GE Healthcare, Madrid, Spain). The non-retained fraction was washed with the same buffer until the absorbance at 280 nm returned to basal levels. The proteases bound to the column were eluted with 100 mM CH_3COONH_4, 1 M NaCl and 25% (v/v) 2-isopropanol, pH 6.5, until the OD at 280 nm was basal. Samples were then dialysed and concentrated by ultrafiltration in Amicon® Ultra 10 K centrifugal filter devices (Millipore, Billerica, MA, USA) and finally stored in 0·15 M PBS at −80 °C until use.

2.2. Fish

Specimens of the turbot *Scophthalmus maximus* (L.), of approximately 50 g body weight, were obtained from a local fish farm. The fish were maintained at 16 °C in 250-L tanks with aerated and recirculated sea water and fed daily with commercial pellets. The fish were acclimatized to the aquarium conditions for two weeks before the start of the experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain). Before all procedures, the fish were anaesthetized with tricaine methanesulfonate (Sigma-Aldrich) (150 mg/L) and killed by pithing.

2.3. Collection of serum, plasma and leukocytes from fish blood

Fish blood was obtained by caudal venous puncture. Serum was obtained from blood that was allowed to clot for 2 h at room temperature before being centrifuged at 3000 x g for 10 min. To obtain the plasma, one ml of blood was mixed with 100 µl of 4% sodium citrate and centrifuged at 3000 x g for 10 min and at 4 °C. Plasma was carefully separated from the cells and used in the coagulation experiments. In some assays, serum and plasma were heated at 45 °C for 30 min. For separation of leukocytes, fish blood was diluted in L-15 medium (1:3, v:v) containing heparin (10 U ml^{-1}) (Castro et al., 1999). The cell suspension was then layered onto a 30%/49% v/v Percoll gradient (GE Healthcare), as previously described (Castro et al., 1999). After centrifugation, the cells at the interface were collected, washed twice in cold L-15 and counted with a haemocytometer. Blood leukocytes (10^7 cells/ml) were then incubated in Eppendorf microtubes with phorbol 12-myristate 13-acetate (PMA) (1 µg/ml) in L-15 medium for 4 h at room temperature to induce the formation of neutrophil extracellular traps (NETs). Control neutrophils were incubated with L-15 medium. NET formation was evaluated under fluorescence microscopy after the leukocyte samples were stained with

SYTOX Green (Thermo Fisher Scientific) (1 μM in tris-buffered saline, TBS) for 30 min and washed carefully with TBS.

2.4. Plasma clotting assays

Pooled fresh plasma from five fish was used in all assays. We tested the procoagulant activity of kaolin (0-200 μg/ml), *P. dicentrarchi* components, including live and lysed ciliates (0-1.3 x 10^6 cells/ml), cilia (0-8000 μg of protein/ml), DNA (0-200 μg/ml), proteases (0-625 μg of protein/ml) and turbot neutrophils (control and treated with PMA, 0-5 x 10^7 cells/ml). With the exception of whole and lysed ciliates and turbot neutrophils, which were resuspended in respectively 3.5% NaCl or L-15 medium, the other samples were diluted in distilled water. Controls containing 3.5% NaCl or L-15 medium were included in the experiment.

Assays were carried out in 2 ml round bottomed microcentrifuge tubes. Test samples (75 μl) and fresh plasma (45 μl) were added to the tubes and mixed. Coagulation was initiated by adding 75 μl of 40 mM $CaCl_2$-solution to the tubes and mixing with the sample and plasma. Finally, the tubes were checked every 10 seconds and coagulation was confirmed by the presence of a rigid clot at the bottom. Results are expressed as mean clotting time for each sample relative to the clotting time for control samples.

2.5. Morphological changes in the ciliates during clot formation

Aliquots (50 μl) of a mixture of ciliates (200 cells), fresh plasma and $CaCl_2$ (at the concentrations mentioned above) were added to probe-clip press-seal incubation chambers (Sigma-Aldrich, Z359459). The changes in ciliate morphology and viability were monitored by differential interference contrast **(**DIC**)** microscopy for at least 30 minutes. In some mixtures, $CaCl_2$ was replaced with 3.5% NaCl and fresh plasma with heated plasma.

2.6. Determination of plasma and serum killing activity in vitro

The killing activity of pooled fresh and heated plasma, fresh and heated serum and, alternatively, plasma treated with the tetrapeptide Gly-Pro-Arg-Pro (Sigma-Aldrich) to prevent clotting (50 μg/mL final concentration), was evaluated in 96 well plates. In all experiments, plasma and serum were obtained from the same group of fish. Plasma and serum were heated to 45 ^oC for 30 minutes. Heating the plasma and serum

inactivates complement and also the coagulation capacity of plasma. In some experiments, we used pooled plasma and serum from 5 immunized fish. The experiment was carried out under aseptic conditions. Ten µl of ciliates (250 ciliates in 3.5 % NaCl), 9 µl of several dilutions of pooled fresh and heated serum or plasma in 3.5% NaCl, 5 µl of 3.5 % NaCl, and 15 µl of 40 mM $CaCl_2$ in distilled water were added to each well (total 39 µl). In some assays, the tetrapeptide Gly-Pro-Arg-Pro was added to 3.5 % NaCl. In addition, $CaCl_2$ was also substituted with 3.5 % NaCl, to prevent clot formation. All components were used at room temperature. The killing activity of serum and plasma was determined by counting the number of dead ciliates per well at several different times. In all experiments triplicate samples were analysed, and the experiment was repeated three times.

2.7. Determination of antibody levels in serum and plasma from immunized fish

To obtain immune serum and plasma, ten fish per group were injected twice, one month apart, with a vaccine containing *P. dicentrarchi* (isolate I1) and the adjuvant Montaine ISA 763 A or PBS, as previously described (Piazzon et al., 2008). One month after injection of the second dose, blood samples were obtained and antibody levels (IgM) were determined by double indirect-ELISA, following the protocol described by Piazzon et al., 2008. Serum and plasma obtained from two groups of five immunized fish were each pooled.

2.8. Analysis of clots and peritoneal exudate with light and scanning electron microscopy in experimentally infected fish

Fish were injected intraperitoneally with 100 µl of PBS containing 10^6 ciliates. At 1, 3 and 6 h post injection, the clots were carefully removed from the peritoneal cavity and fixed in 10% buffered formalin, for analysis by light and fluorescence microscopy, or with cold 2% paraformadehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, for analysis by scanning electron microscopy (SEM). For light and fluorescence microscopy, clots were cryoprotected with 30% sucrose in PBS, embedded in OCT compound (Tissue Tek, Torrance, CA), frozen with liquid nitrogen-cooled isopentane and sectioned on a cryostat. Sections were stained with 4',6-diamidino-2-phenylindole (DAPI) (100 µM, 10 min) or haematoxylin and eosin (H&E). Other clots were added directly to slides and examined by differential interference contrast (DIC) microscopy. In addition, the peritoneal cavity was washed with cold L-15 medium and

heparin (10 U ml^{-1}). One drop of cell suspension from each sample was placed on a slide and examined directly by DIC microscopy or was stained with SYTOX green and observed by fluorescence microscopy. Other samples were placed on a slide, dried with a hair drier and stained with Haemacolor (Merck) or diaminobenzidine (Sigma–Aldrich) (for peroxidase activity) (Kiernan, 1981). Alternatively, cell smears were fixed for 30 min at 4 °C in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and postfixed for 30 min in 1% osmium tetroxide in the same buffer. The samples were then washed three times in dH_2O, dehydrated with a series of graded ethyl alcohols, chemically dried with hexamethyldisilazane (HMDS) and sputter-coated with iridium, before finally being observed and photographed in an Ultra Plus Zeiss scanning electron microscope.

2.9. CD11b and CD18 gene expression in peritoneal cells

Thirty fish (five fish per group) were injected intraperitoneally with 100 µl of PBS or with PBS containing 10^6 ciliates. At 1, 3 and 6 h post injection, the peritoneal cavity was washed as indicated above. The cell suspensions thus obtained were washed twice with PBS, and the pellets obtained after centrifugation were frozen in liquid nitrogen and held at −80 °C until RNA extraction. RNA was extracted using the RNAeasy Mini kit (Qiagen) according to the manufacturer's recommendations. Genomic DNA contamination was removed from RNA samples with DNase I (Thermo Scientific, Surrey, UK.). RNA quality and quantification were evaluated respectively in a Bioanalyzer (Bonsai Technologies) and in a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc.), as indicated by Fontenla et al., (2016). cDNA was synthesized using the cDNA synthesis kit (NZYTech, Portugal) with 1 µg of sample RNA. qPCR was performed with gene-specific primers for the CD11b gene (forward/reverse primer pair CD11bF/CD11bR, 5′-AGGTTCATGGGAAGACTGGA-3′/5′-ATTGGACCCTGCTGAAAAGA-3′), and for the CD18 gene (forward/reverse primer pair CD18F/CD18R, 5′-AGAACCACCCAGCGTCATAG-3′/5′-TTGCCCACTTGGATTTCTTC-3′). Elongation factor 1-alpha (ef1-α) was used as a housekeeping gene for qPCR analysis, by including the forward/reverse primer pair 5′-GGAGGCCAGCTCAAAGATGG-3′/5′-ACAGTTCCAATACCGCCGATTT-3′. The Primer 3Plus program was used with default parameters to design and optimize the primer sets. The qPCR reaction was performed with a NZY qPCR Green Master Mix (NZYTech, Portugal). The primer pair for the genes under study was used at a final

concentration of 300 nM. The volume was completed with 1 µL of cDNA and RNase free distilled H2O to a final reaction volume of 10 µL/well. Quantitative PCR was conducted at 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s, 60 ºC for 30 s, and 72 ºC for 30 s, ending with a melting-curve analysis at 95 ºC for 15 s, 55 ºC for 15 s and 95 ºC for 15 s. All quantitative PCRs were performed in a CFX Connect™ Real-Time PCR Detection System (BioRad). The relative quantification of gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) applied with software conforming to minimum information for publication of qRT-PCR experiments (MIQE) guidelines (Bustin et al., 2009).

2.10. Statistics

Results shown in the figures are expressed as means ± standard error. Significant differences ($P \leq 0.05$) were determined by analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test.

3. Results

3.1. Intraperitoneal injection of P. dicentrarchi *in turbot generates the formation of internal clots*

In previous studies, we observed plasma clots in the peritoneal cavity of turbot injected intraperitoneally with the fish pathogen *P. dicentrarchi*. The clots contained ciliates, suggesting the involvement of the coagulation system in fish defence against this ciliate. To confirm those observations, we injected 36 turbot (12 per sampling time) with ciliates and sampled them after 1, 3 and 6 h. Macroscopic clots of different sizes were observed in the peritoneal cavity of 34 of the fish (Fig. 1A, B), and no living ciliates were found in the peritoneal cavity of 14 of the fish at the different sampling times. The clots included areas with ciliates and areas with a mixture of peritoneal cells and ciliates (Figs. 2 and 3). The ciliates located inside the clots were immobilized and many of them were lysed (Fig. 4). Ciliates outside the clots were usually alive, although dead ciliates were also observed outside of the clots in the peritoneal cavity of some fish.

3.2. Plasma clots immobilize and kill the ciliates in vitro

To determine the effects of fresh plasma on *P. dicentrarchi*, we mixed the ciliates with citrated plasma and then induced clotting by adding $CaCl_2$ to the mixture.

Under these conditions, ciliates were gradually immobilized inside the clots and usually became increasingly elongated until cell death occurred (Fig. 5A, B). In the absence of clotting, plasma also killed the ciliate. In this case, as after incubation with serum, ciliates became rounded and finally lysed (Fig. 5C, D). We also evaluated the killing activity of serum and plasma from naive and immunized fish in the presence and absence of $CaCl_2$. Plasma from naive fish disaplyed higher killing activity than serum. The addition of $CaCl_2$ clearly increased the killing activity of both plasma and serum (Fig. 6A, B). The killing activity of plasma and serum from vaccinated fish (with specific IgM detectable by ELISA) was similar and much higher than in the samples from naive fish. Adding $CaCl_2$ also increased the killing activity of both immune plasma and serum (Fig. 6C, D). In previous studies, we have shown that the killing activity of serum mainly involved complement. We heated the serum in order to inactivate the complement; however, heating also inactivates plasma clotting and it is not possible to distinguish the killing due to complement and that due to clotting. To test the importance of plasma clotting in ciliate killing, we inhibited the process by adding the tetrapeptide Gly-Pro-Arg-Pro, which inhibits fibrinogen/thrombin clotting in mammals. We first determined the inhibitory concentrations of the tetrapeptide in plasma samples containing $CaCl_2$ and then compared the killing activity of plasma with or without the tetrapeptide (at a final concentration of 50 μg/mL). Interestingly, the results show that plasma tended to kill *P. dicentrarchi* slightly faster when clotting was inhibited by the tetrapeptide, although the total mortality of ciliates was similar (Fig. 7).

3.3. P. dicentrarchi, *several ciliate components and PMA-stimulated neutrophils display procoagulant activity* in vitro

As intraperitoneal injection of *P. dicentrarchi* generated the formation of clots in the peritoneal cavity of injected fish, we tested the capacity of whole ciliates or several ciliate components to induce plasma clotting *in vitro*. We first tested kaolin, a particulate activator that induces activation of the contact pathway in mammals, as an accelerator of plasma clotting in turbot. Pooled citrated plasma was previously mixed with kaolin, and clotting was induced by adding $CaCl_2$ to the mixture. The time until clotting was then determined. In the absence of kaolin, fresh turbot plasma clotted in about 5 min. The presence of kaolin greatly reduced the plasma clotting time, and clotting occurred within one minute at the maximum concentration used (200 μg/ml) (Fig. 8A). We then tested the procoagulant activity of living ciliates, ciliates lysed by

sonication and several ciliate components (cilia, proteases and DNA). We found that living ciliates greatly reduced the plasma clotting time, and this effect was observed at ciliate concentrations higher than $1x10^4$ ciliates per mL (Fig. 8A). Lysed ciliates had a weaker effect than living ciliates, and a concentration of about $8x10^4$ ciliates per mL was required before a reduction in clotting time was observed (Fig. 8A). Cilia and ciliate DNA also displayed procoagulant activity (Fig. 8B), and concentraions of respectively 60 μg/ml and 1.5 μg/ml were required to observe an effect on clotting. Low concentrations of ciliate proteases tended to increase the clotting time, and high concentration clearly reduced the plasma clotting time (Fig. 9A). Some studies have suggested that NETs can activate the intrinsic pathway of coagulation in mammals. We induced NETs in turbot blood neutrophils activated with PMA and tested the effect on plasma clotting. Concentrations of PMA-treated neutrophils higher than $2x10^5$ cells per mL reduced the clotting time (Fig. 9B). By contrast, untreated neutrophils only showed some procoagulant activity at very high concentrations.

3.4. Living ciliates induced the formation of large aggregates of neutrophils in the turbot peritoneal cavity

The injection of ciliates generated a massive release of neutrophils to the peritoneal cavity. The presence of live ciliates in the peritoneal cavity tended to lead to aggregation of neutrophils and the formation of large groups of cells (Figs. 10A, B). In fish in which all the ciliates were killed, neutrophils were also very abundant in the peritoneal cavity, although they did not form large aggregates of cells (Fig. 11). In infected fish, in addition to the presence of clots, abundant microscopic fibrin-like fibres of different sizes were observed in the peritoneal exudate (Fig. 12). We observed, by SEM, numerous fibrin-like fibres in the exudate, many of which were associated with peritoneal leukocytes, possibly neutrophils (Figs. 13A, B), and with ciliates (Fig. 13C).

As we found that PMA-treated neutrophils accelerated coagulation, we looked for the presence of NETs in the neutrophil aggregates. We observed NETs in very few cells (Fig. 14) and no extracellular DNA in most of the free-living cells or cell aggregates. Finally, we determined the expression of α (CD11b) and β (CD18) integrin chains, which are genes associated with fibrin deposition on neutrophils and play an important role in cell adhesion in mammals. Interestingly, we observed a substantial increase in the expression of both genes in peritoneal cells of fish injected with *P. dicentrarchi* (Fig. 15).

4. Discussion

Blocking the entry of pathogens is important to prevent their dissemination throughout the body. The coagulation system has been shown to participate in immunity in invertebrates, and it is capable of immobilizing and killing bacteria (Iwanaga and Lee, 2005; Theopold et al., 2014); however, the genes involved in coagulation are not considered homologous to those found in vertebrates. In the present study, clots formed in the peritoneal cavity of turbot injected with *P. dicentrarchi*, as early as 1 h post injection, and many of the clots contained numerous immobilized ciliates. We also found that the coagulation system is important in the turbot defence against this pathogen. The presence of these clots at the injection site suggest that the ciliates activated the coagulation system and that this system, in addition to its role in haemostasis, may play a role in the defence against pathogens, as also found in mammals (Gaertner and Massberg, 2016). The mammalian coagulation system can be activated by two different pathways, both of which generate clot formation: the tissue factor (extrinsic) pathway, which becomes activated following damage to blood vessels, and the contact (intrinsic) pathway, which becomes activated after contact with anionic surfaces (Berends et al., 2014; Smith et al., 2015). Both extrinsic and intrinsic pathways are considered part of host defence against infection (van der Poll and Herwald, 2014). With the data obtained in the present study, we cannot determine which pathway became activated in turbot after injection with *P. dicentrarchi* and participated in clot formation. Although components of the extrinsic pathway have been found in fish genomes (Jiang and Doolittle, 2003), their role in the fish immune response has not yet been established. Unfortunately, there is not much information about the existence of a contact pathway in fish. This system contributes to host defence, via the proinflammatory kallikrein-kinin system, and coagulation, via the procoagulant intrinsic coagulation pathway. The principal initiator is factor XII (FXII), which becomes activated by bacterial surfaces, fungi and even viruses (Long et al., 2016). However, several components of the this pathway have not been identified in teleosts, including factor FXII and plasma kallikrein (Doolittle 2011; Wong and Takei, 2013;Yin et al., 2016), and some authors have suggested that fish lack the intrinsic pathway (Papareddy et al., 2018). We have carried out some functional assays in order to shed more light on this subject. We induced plasma clotting in turbot by using some of the compounds also used in mammals to stimulate the contact pathway, such as kaolin, DNA and neutrophil

NETs (Kannemeier et al., 2007). We found that all of these compounds accelerated plasma clotting *in vitro*, suggesting the existence of a contact coagulation system in turbot. As FXII and plasma kallikrein are absent in fish, the contact pathway must be activated by other compounds. A kalliklectin with structural similarities to mammalian plasma kallikreins and coagulation factor XI has been described in some fish species. It has been suggested that the mammalian enzymes may have originally emerged as a lectin and then evolved into molecules with protease activity after separation from common ancestors (Tsutsui et al., 2011). However, it is not known whether a lectin participates in the activation of a pathway alternative to the tissue factor pathway. Further work is required to establish which pathways are involved in the induction of clots by *P. dicentrarchi* in vivo.

We also investigated the plasma coagulation activity induced by *P. dicentrarchi* and ciliate components. Interestingly, living ciliates are also good activators of this response, and they are more effective than lysed ciliates. Numerous pathogens, including bacteria, viruses, fungi and some parasites can activate the coagulation system in mammals (Nickel and Renné, 2012). As far as we know, the present study is the first to report the capacity of ciliates to induce plasma clotting in vertebrates. Because this process occurs relatively rapidly *in vitro* and at low ciliate concentrations, the clotting must be accelerated by something on the ciliate surface, and the movement of cilia may even have some effect. We found that cilia alone also displayed procoagulant activity, but at a much lower level than living ciliates. Other parasite components also reduced the clotting time *in vitro*; this is the case with *P. dicentrarchi* proteases, which are considered important virulence factors in this ciliate (Paramá et al., 2007a,b; Piazzon et al., 2011b). The pathogen proteases have diverse effects on plasma coagulation. Some bacteria escape fibrin(ogen) meshes by inducing proteolytic dissolution, while proteases from other species have the opposite effect (Dubin et al., 2013). Something similar occurs in parasites. Serine proteases from parasitic helminths display anticoagulation activity (Yang et al., 2015). Although overall the *P. dicentrarchi* proteases display procoagulant activity, other complex groups of proteases may be involved, and each may have different effects.

In order to evaluate the importance of clotting in killing *P. dicentrarchi*, we treated the plasma with the tetrapeptide Gly-Pro-Arg-Pro, which inhibits fibrin polymerization in mammalian plasma (Pitkänen et al., 2017). We found that this tetrapeptide also inhibits plasma clotting in turbot. Interestingly, there was a delay in *P.*

dicentrarchi killing in plasma samples in which clotting occurred relative to that in plasma treated with the tetrapeptide, in which clotting was inhibited. In previous studies, we have found that turbot complement is highly effective in killing *P. dicentrarchi* (Leiro et al., 2008; Piazzon et al., 2011a). Plasma clotting may restrict the movement of ciliates, but clotting may also restrict the access of complement components to the ciliates, thereby reducing the speed of ciliate killing by plasma. Moving ciliates probably come into contact with more complement than immobile ciliates. Nonetheless, clotting clearly blocks ciliate movement, and the space occupied by the ciliate gradually becomes smaller until cell death occurs.

We know from previous studies that turbot complement, especially after activation by the classical pathway, is highly effective in killing *P. dicentrarchi* (Leiro et al., 2008). In the present study, we compared the killing activity of plasma and serum from naive and vaccinated fish. In naive fish, plasma displayed higher killing activity than serum in the absence of clotting. When clotting was induced in plasma, we found that the addition of calcium to plasma and serum from naive fish greatly increased the killing activity. Under these conditions, plasma also displayed higher killing activity than serum. It is not clear why the addition of calcium should increase the killing activity of naive serum. Previous studies have shown that calcium is required for activation of the classical complement pathway in fish, but not for the activation of the alternative pathway (Yano, 1992). Calcium chelation did not inhibit the killing activity of sea bream (*Sparus aurata*) serum through the alternative complement pathway (Sunyer and Tort, 1995). On the basis of these results, the addition of calcium to plasma from naive turbot should not affect the activity of the alternative complement pathway. However, we cannot rule out the activation of the classical pathway by natural antibodies present in plasma and serum. Differences in killing activity between plasma and serum from naive turbot in the presence of calcium may be associated with activation of the coagulation system, as it has been shown that components of this system can also trigger complement activation in mammals (Irmscher et al., 2018; Kaplan and Ghebrehiwet, 2010). Activation of the coagulation system in turbot may enhance complement activation on the ciliate surface, increasing killing. Differences in killing activity of serum and plasma from immunized fish were less evident than in samples from naive fish. The main reason for this may be that turbot complement is highly effective in killing *P. dicentrarchi* when activation occurs through the classical pathway (Leiro et al., 2008; Piazzon et al., 2011a), and the influence of the activation of

coagulation system on this activity may be more difficult to detect. All of these aspects must be evaluated in future research, to confirm or rule out the interactions between complement and the coagulation system in fish and their influence on killing activity of pathogens.

In addition to the formation of plasma clots, we found that a large number of neutrophils were released to the peritoneal cavity as a consequence of i.p. injection of *P. dicentrarchi*. These neutrophils were highly stimulated and formed very large cell aggregates. In addition to neutrophils, we observed abundant fibrin-like fibres in the peritoneal exudate. The fibres were often associated with neutrophils and ciliates. In response to infectious stimuli, neutrophils can release serine proteases and also NETs, which help to kill the pathogens. NETs and serine proteases promote coagulation and fibrin deposition and prolong lysis of clots (Longstaff et al., 2013; Martinod and Wagner, 2016; Pheiler et al., 2017). Stimulation of teleost neutrophils leads to production of NETs (Havixbeck and Barreda, 2015). In the present study, we found that injection of *P. dicentrarchi* induces the formation of NETs in some of the neutrophils that formed part of the aggregates. However, as this process only affected some neutrophils, and most of them did not show alterations compatible with NETs, it probably does not explain the large amount of fibrin-like fibres generated in the peritoneal exudate. We observed strong upregulation of the expression of CD11b and CD18 in the peritoneal leukocytes from infected turbot. The findings of mammalian studies have suggested that fibrin deposition on neutrophils is markedly reduced by antibodies against the integrin CD11b/CD18 (also known as CR3 or Mac-1) (Goel and Diamond, 2001). CD11b/CD18 can bind to several molecules, including fibrin(ogen) (Flick et al., 2004), and CD11b displays procoagulant activity (Gorbet and Sefton, 2001). Peritoneal nodules have been shown to form in the peritoneal cavity of vaccinated turbot (Noia et al., 2014) and also in mice injected with aluminium hydroxide (Munks et al., 2010). In the latter, fibrinogen was critical for nodule formation, and the process involved $CD11b^+$ cells. In addition, neutrophils were the primary cell type involved in fibrin formation (Munks et al., 2010). All of these aspects must be investigated in greater detail; however, on the basis of the findings in mammals and the presence of numerous fibrin-like fibres associated with turbot leukocytes, it appears that clotting in the turbot peritoneal cavity after injection of *P. dicentrarchi* may be influenced by the presence of the integrin CD11b/CD18 in peritoneal leukocytes and particularly in neutrophils.

In conclusion, the findings of the present study show an interaction between *P. dicentrarchi* and the fish coagulation system: the ciliate can induce clotting, which in turn affects the viability of the ciliate. To analyse these interactions in greater detail, new tools must be developed to enable evaluation of the response at the molecular level.

Acknowledgements

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Figures

Fig. 1. Plasma clots (arrow) obtained from the peritoneal cavity of infected turbot at 1h (A) and at 6 h (B) post injection.

Fig. 2. Cryostat section of a plasma clot stained with DAPI, showing areas of ciliates (yellow arrow) and areas of fish cells (orange arrow).

Fig. 3. Haematoxylin and eosin (H&E) stained section of a plasma clot showing an area rich in ciliates (arrows).

Fig. 4. Plasma clot containing numerous ciliates (arrows), some of them lysed or with altered morphology (yellow arrows). Some ciliates with normal morphology are also observed outside the clot (red arrow). DIC microscopy.

Fig. 5. Sequence of images showing *P. dicentrarchi* in a plasma clot generated in vitro (A and B). Note the changes in ciliate morphology. Ciliates became elongated and finally fragmented. DIC microscopy. A ciliate incubated with citrated plasma without $CaCl_2$, which showed spherical morphology before undergoing lysis (C), and a control ciliate (D) are also observed. Light microscopy.

Fig. 6. Ciliate mortality (%) after incubation of *P. dicentrarchi* with naive or immune plasma and serum, in the presence and absence of $CaCl_2$, for 30 min, 1 or 3 h. When naive fish were used, plasma generated higher killing activity than serum in the presence (A) and absence of $CaCl_2$ (B). In immune fish, the killing activity of serum and plasma increased substantially, but with no differences in killing activity (C and D).

Fig. 7. Ciliate mortality (%) after incubation of *P. dicentrarchi* with plasma, in the presence or absence of the tetrapeptide Gly-Pro-Arg-Pro (50 μg/mL). When $CaCl_2$ was added, the presence of peptide increased plasma killing activity during the first 30 min of incubation. The peptide alone had no effect on ciliate mortality (not shown in the graph). *$P<0.05$ compared with plasma and $CaCl_2$.

Fig. 8. The procoagulant activity of kaolin, living ciliates and ciliate components in pooled turbot plasma. The procoagulant activity of living and lysed ciliates (A) and of cilia and ciliate DNA (B) is compared with the activity of kaolin. Results are shown as the clotting time (s) for each experimental sample concentration relative to that observed for the control samples (0 s, blue arrows). A negative or positive value indicates that the clotting time of experimental samples was respectively lower or higher than that of the control samples.

Fig. 9. Procoagulant activity of kaolin, ciliate proteases, neutrophils or neutrophils preincubated with PMA. The procoagulant activity of ciliate proteases (A) and of neutrophils and neutrophils preincubated with PMA (B) was compared with the kaolin activity. Results are shown as the clotting time (s) for each concentration of test sample relative to that of the control sample (0 s, blue arrows). Negative and positive values indicate that the clotting time in the test samples was respectively lower or higher than in the control samples.

Fig. 10. Turbot peritoneal cells 6 h after injection with *P. dicentrarchi*. A) Several large neutrophil aggregates (blue arrows) are observed. These fish contained living ciliates in the peritoneal cavity. Ciliates (red arrow). B) Detail of neutrophil aggregates with several ciliates feeding on them. Ciliates contain several phagocytosed, peroxidase-positive neutrophils. Smear of peritoneal fluid stained with peroxidase-haematoxylin.

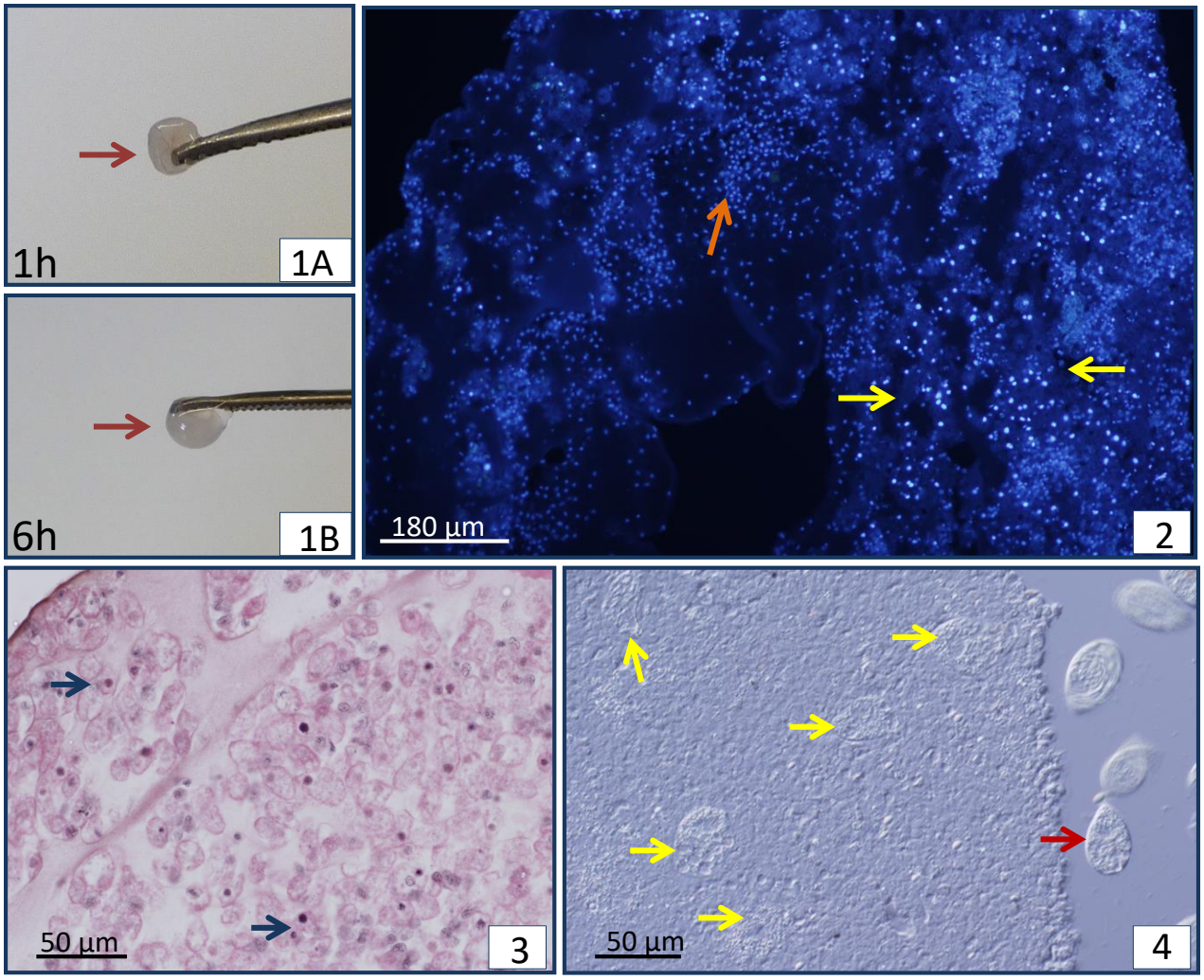
Fig. 11. Turbot peritoneal cells 6h after injection with *P. dicentrarchi*. The fish contained dead ciliates in the peritoneal cavity. Numerous neutrophils (peroxidase positive cells, blue arrows) were observed but not aggregated. Smear of peritoneal fluid stained with peroxidase-haematoxylin.

Fig. 12. Smear of turbot peritoneal fluid 1 h after injection with *P. dicentrarchi* and showing abundant fibrin-like fibres (arrows). Neutrophil aggregate (N). Peroxidase-haematoxylin.

Fig. 13. Scanning electron microscopy images of peritoneal exudate from turbot 6 h after injection with *P. dicentrarchi*. A) Numerous fibres are observed between the cells (arrows) and some are attached to peritoneal leukocytes. B) Several fibres are attached to a peritoneal leukocyte (arrows) or, C) to a ciliate (arrows).

Fig. 14. Peritoneal cell aggregate obtained 6 h after injection of fish with *P. dicentrachii* and stained with SYTOX Green. The morphology of very few cells was compatible with release of NETs (arrow) and most cells did not have extracellular DNA. Fluorescence microscopy.

Fig. 15. Expression profiles of CD11b and CD18 in peritoneal cells 1, 3 and 6 after injection with *P. dicentrarchi* or PBS. The expression was calculated relative to the ef1-α mRNA level. Data are means±SEM for five fish. The asterisks denote statistically significant differences($P<0.05$) between fish injected with PBS (C, control group) and fish injected with ciliates (I).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1A
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180 µm
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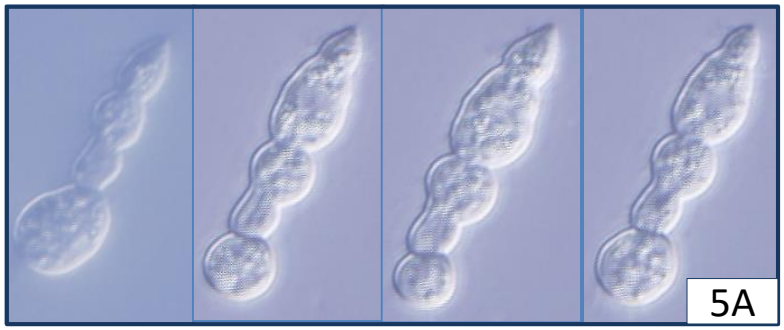
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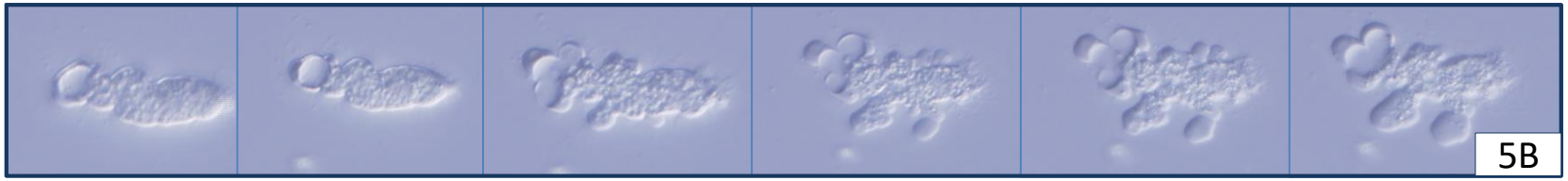
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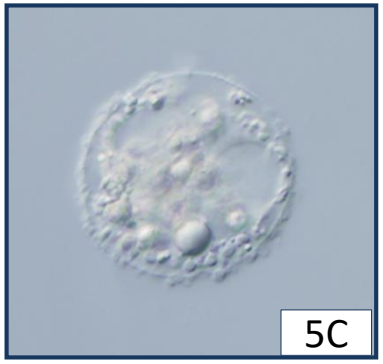
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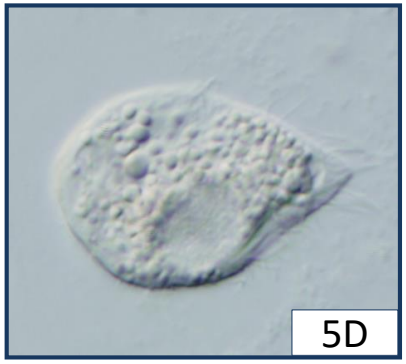
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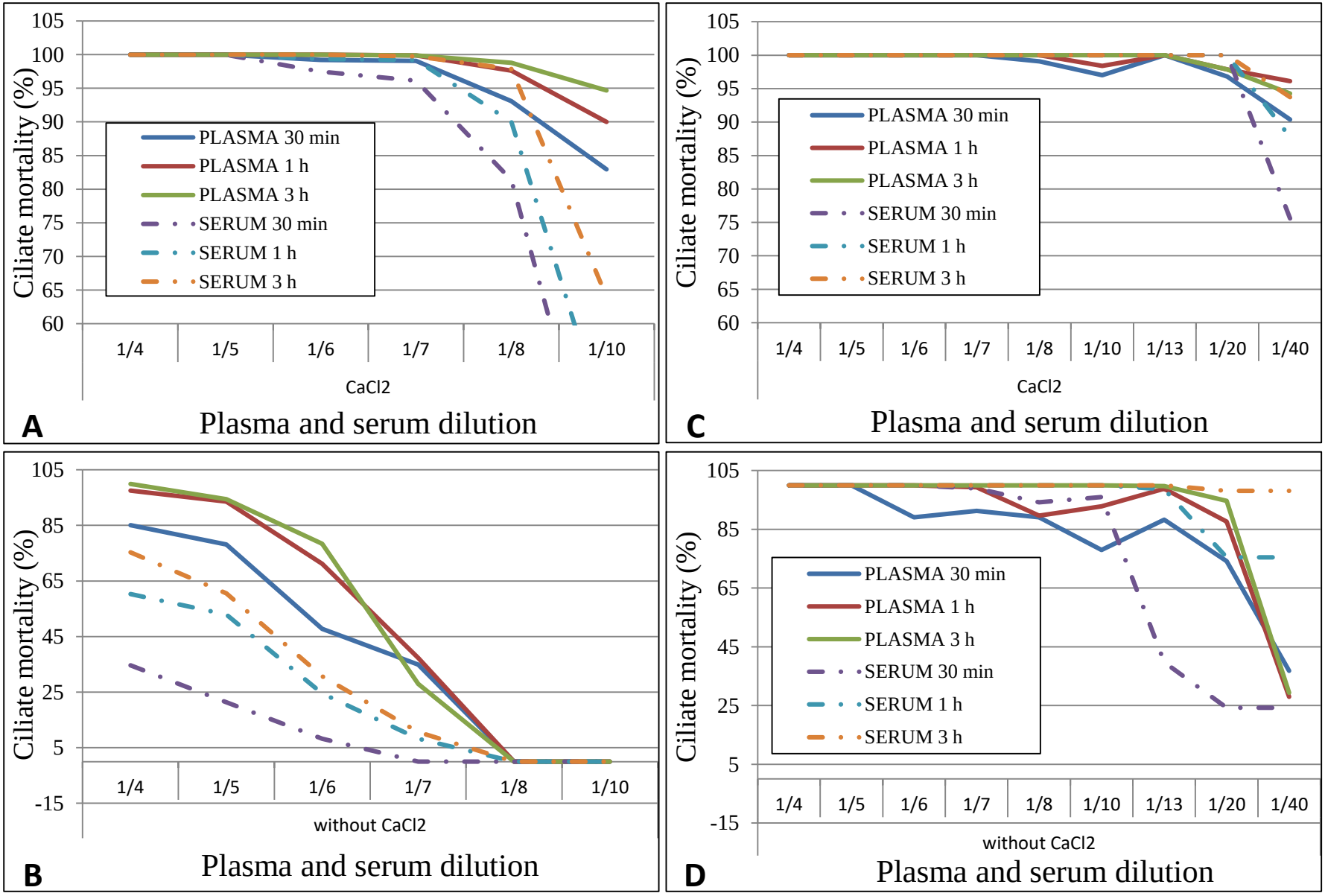

Ciliate mortality (%)
105
100
95
90
85
80
75
70
65
60
PLASMA 30 min
PLASMA 1 h
PLASMA 3 h
SERUM 30 min
SERUM 1 h
SERUM 3 h
1/4
1/5
1/6
1/7
1/8
1/10
CaCl2
A
Plasma and serum dilution
Ciliate mortality (%)
105
85
65
45
25
5
-15
1/4
1/5
1/6
1/7
1/8
1/10
without CaCl2
B
Plasma and serum dilution
Ciliate mortality (%)
105
100
95
90
85
80
75
70
65
60
PLASMA 30 min
PLASMA 1 h
PLASMA 3 h
SERUM 30 min
SERUM 1 h
SERUM 3 h
1/4
1/5
1/6
1/7
1/8
1/10
1/13
1/20
1/40
CaCl2
C
Plasma and serum dilution
Ciliate mortality (%)
105
85
65
45
25
5
-15
PLASMA 30 min
PLASMA 1 h
PLASMA 3 h
SERUM 30 min
SERUM 1 h
SERUM 3 h
1/4
1/5
1/6
1/7
1/8
1/10
1/13
1/20
1/40
without CaCl2
D
Plasma and serum dilution

Fig. 6

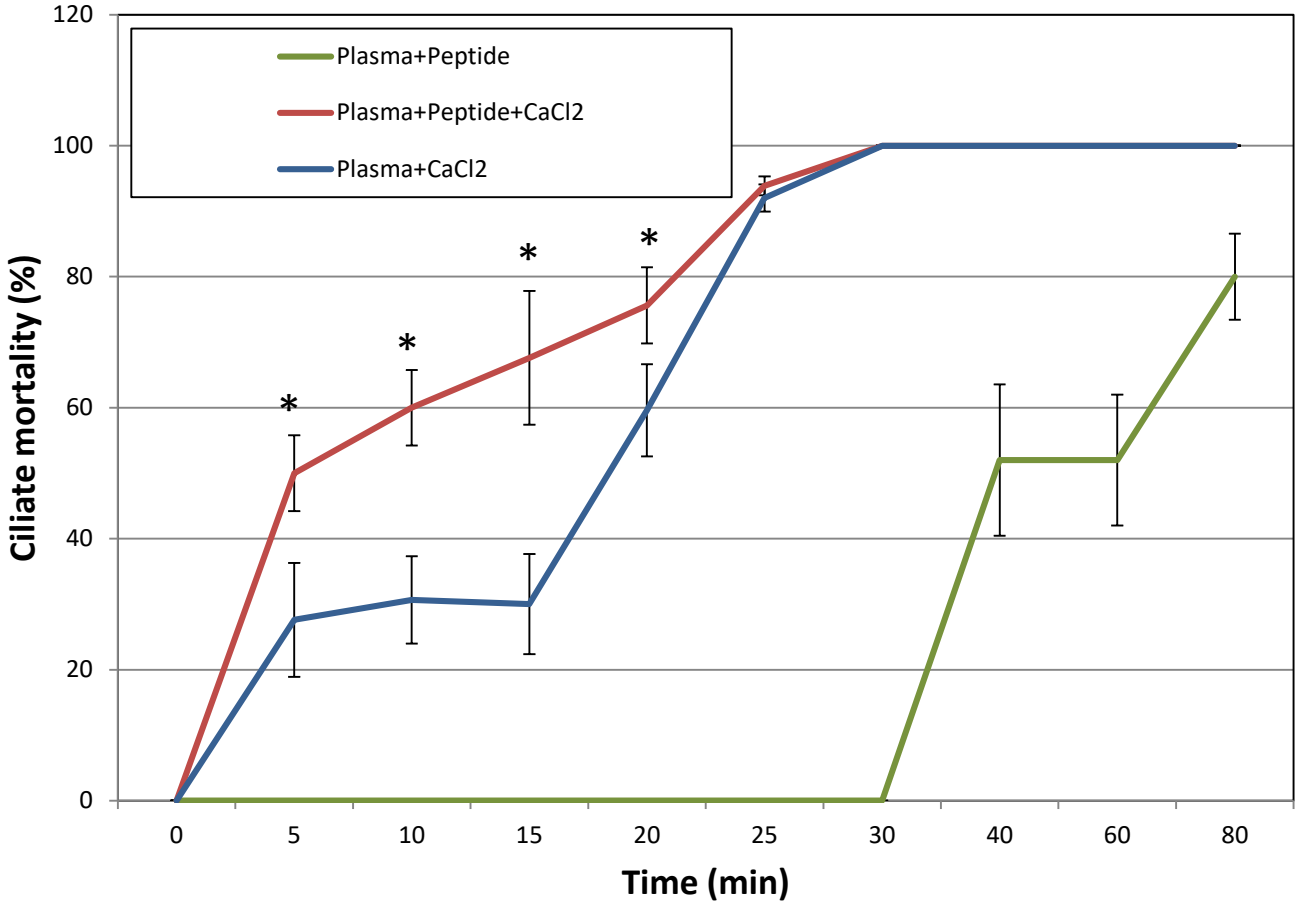
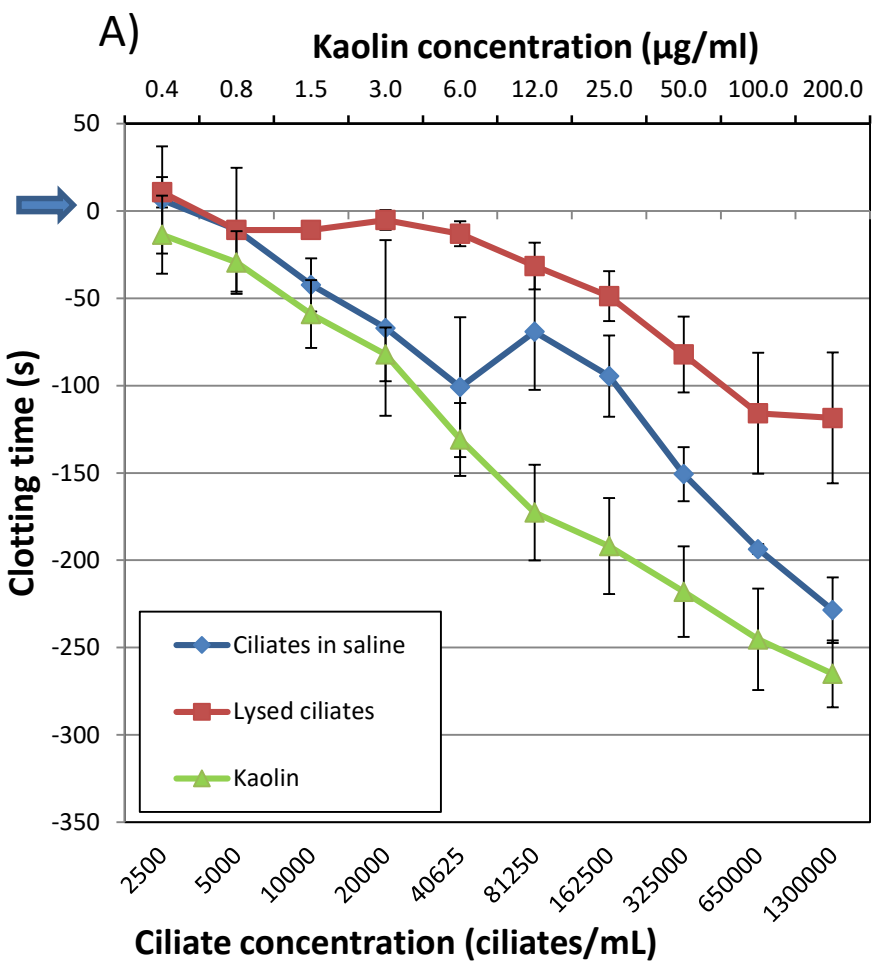

Plasma+Peptide
Plasma+Peptide+CaCl2
Plasma+CaCl2
Ciliate mortality (%)
Time (min)
0
20
40
60
80
100
120
0
5
10
15
20
25
30
40
60
80
*
*
*
*

Fig. 7


A)
Kaolin concentration (µg/ml)
0.4
0.8
1.5
3.0
6.0
12.0
25.0
50.0
100.0
200.0
50
0
-50
-100
-150
-200
-250
-300
-350
Clotting time (s)
Ciliates in saline
Lysed ciliates
Kaolin
2500
5000
10000
20000
40625
81250
162500
325000
650000
1300000
Ciliate concentration (ciliates/mL)

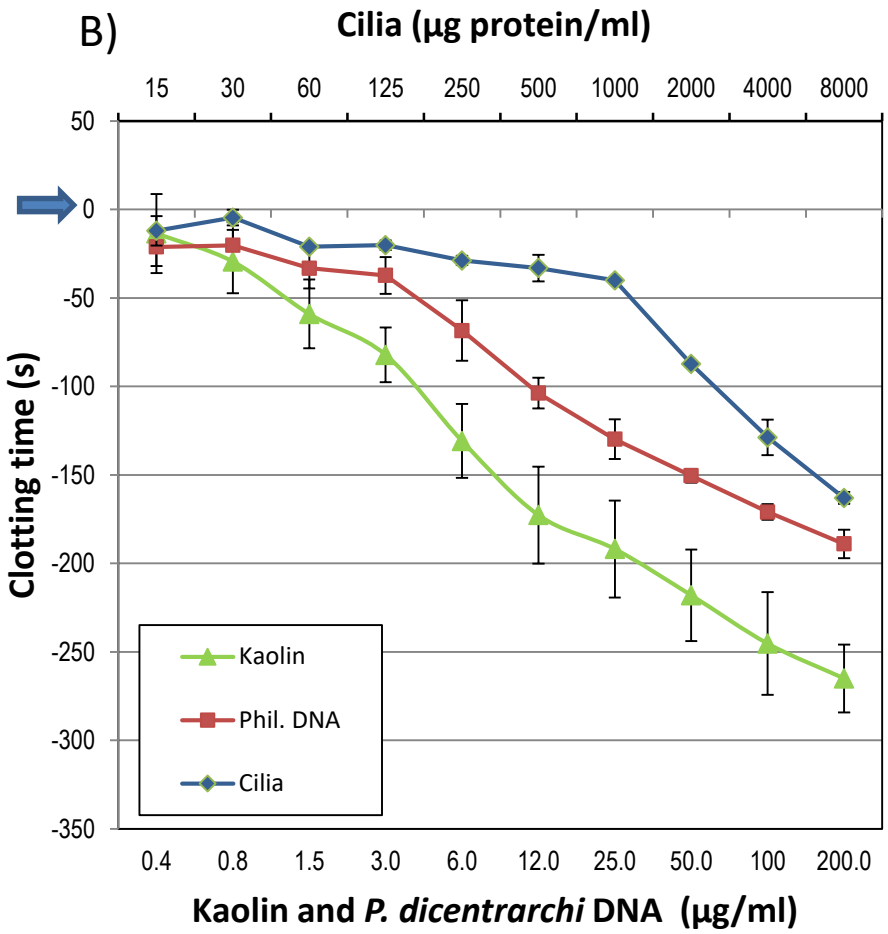
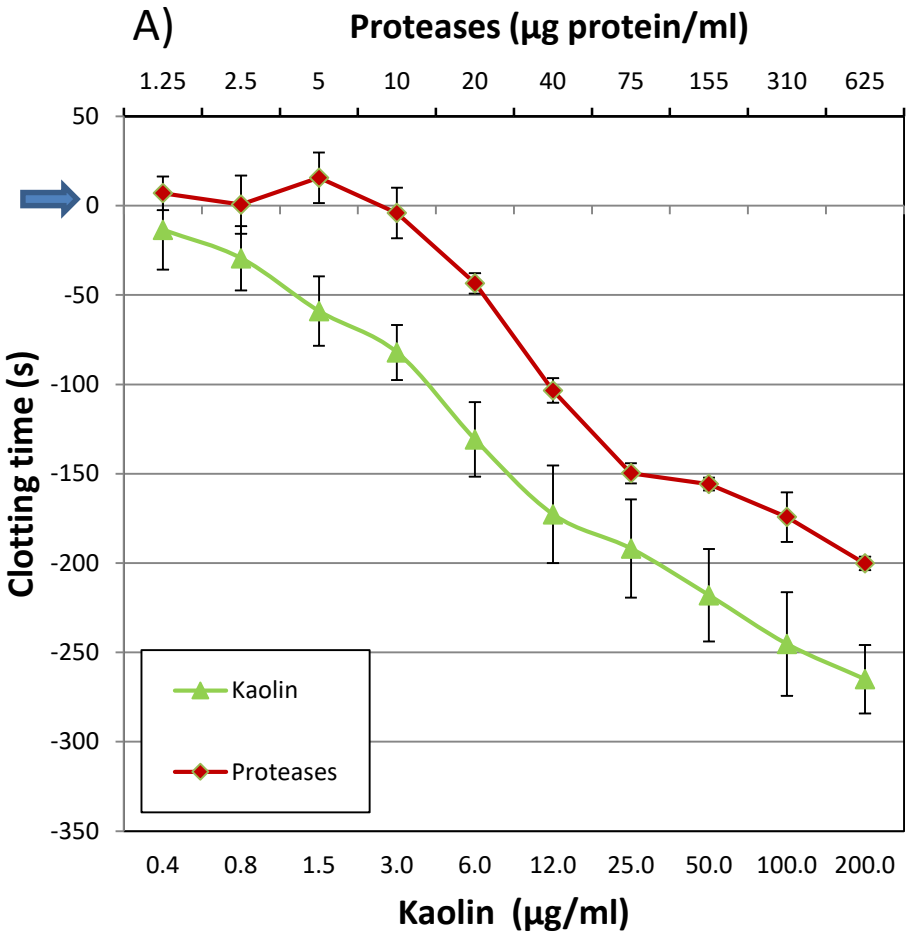

B)
Cilia (µg protein/ml)
15
30
60
125
250
500
1000
2000
4000
8000
50
0
-50
-100
-150
-200
-250
-300
-350
Clotting time (s)
Kaolin
Phil. DNA
Cilia
0.4
0.8
1.5
3.0
6.0
12.0
25.0
50.0
100
200.0
Kaolin and P. dicentrarchi DNA (µg/ml)

Fig. 8


A)
Proteases (µg protein/ml)
1.25
2.5
5
10
20
40
75
155
310
625
50
0
-50
-100
-150
-200
-250
-300
-350
Clotting time (s)
Kaolin
Proteases
0.4
0.8
1.5
3.0
6.0
12.0
25.0
50.0
100.0
200.0
Kaolin (µg/ml)

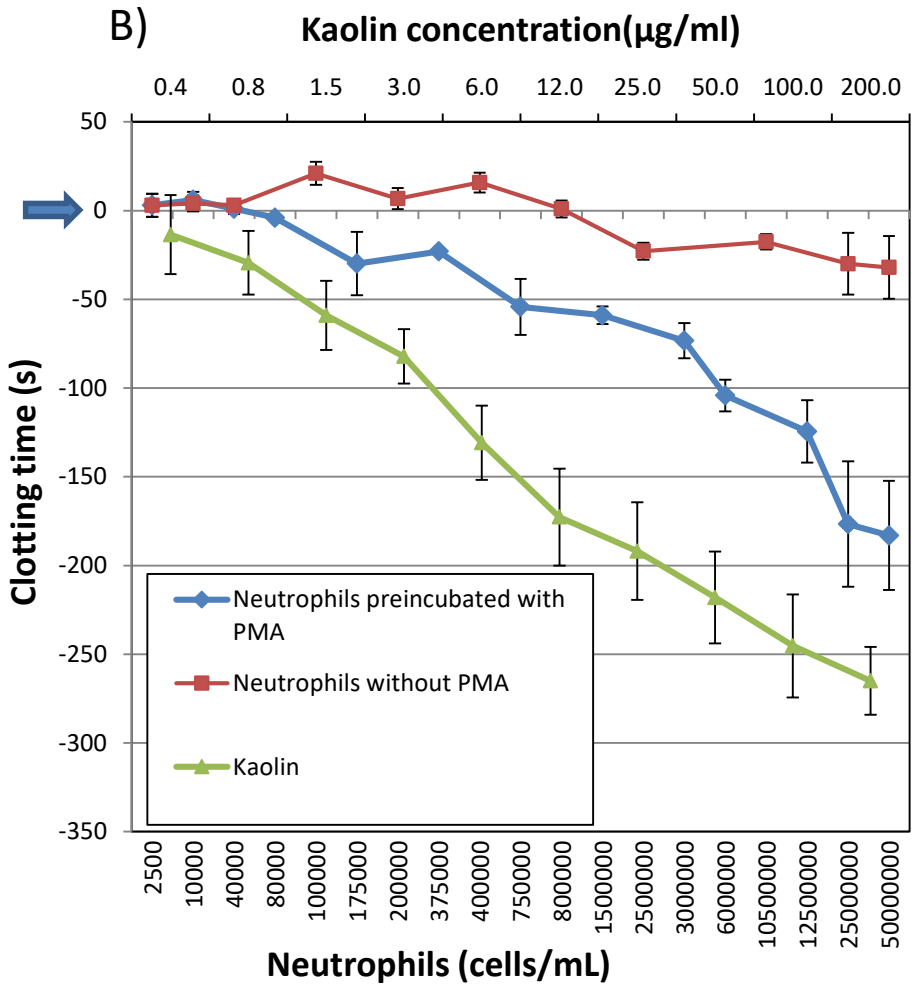
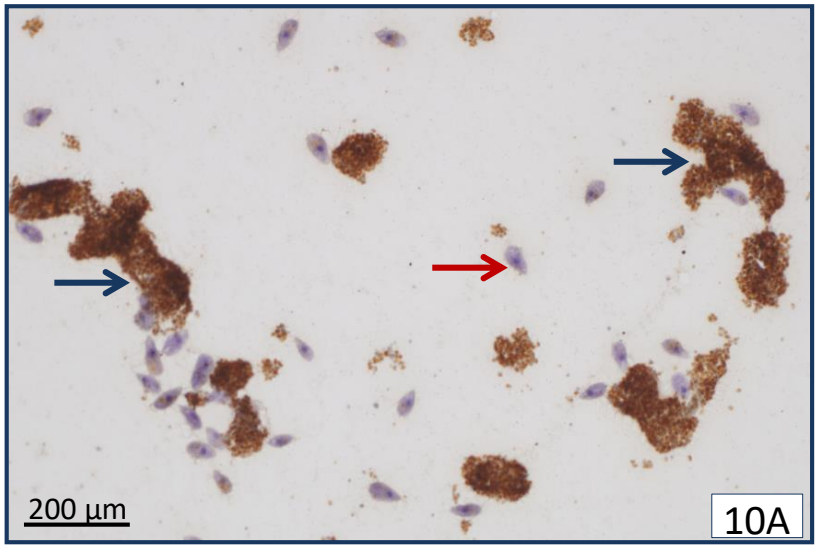
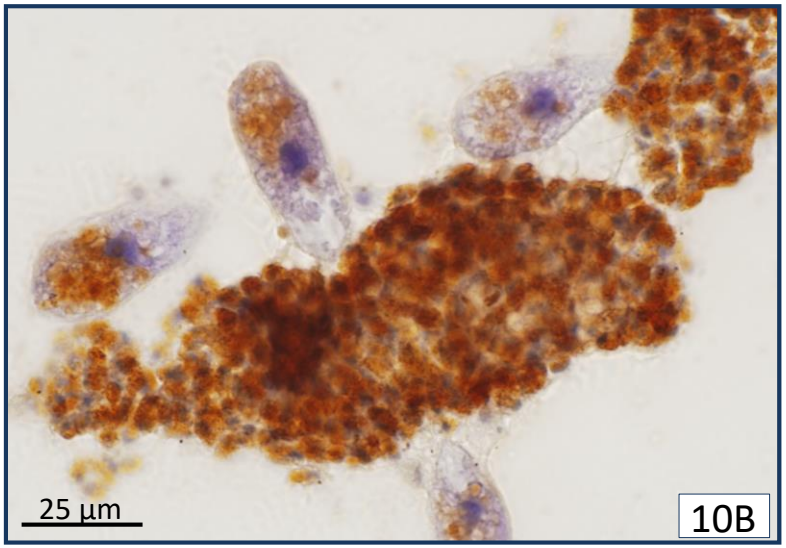
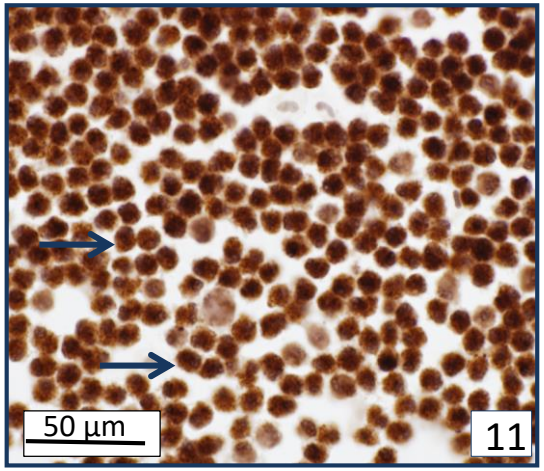
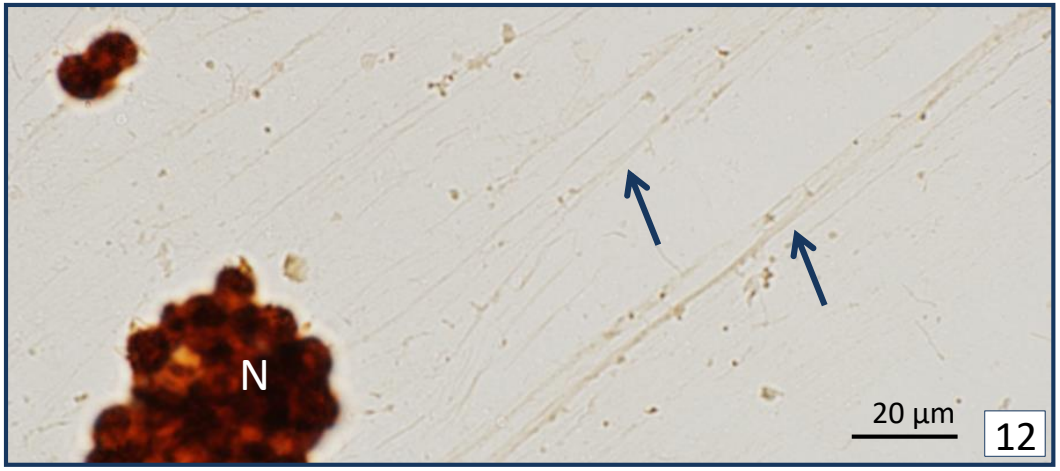

B)
Kaolin concentration(µg/ml)
0.4
0.8
1.5
3.0
6.0
12.0
25.0
50.0
100.0
200.0
50
0
-50
-100
-150
-200
-250
-300
-350
Clotting time (s)
Neutrophils preincubated with PMA
Neutrophils without PMA
Kaolin
2500
10000
40000
80000
100000
175000
200000
375000
400000
750000
800000
1500000
2500000
3000000
6000000
10500000
12500000
25000000
50000000
Neutrophils (cells/mL)

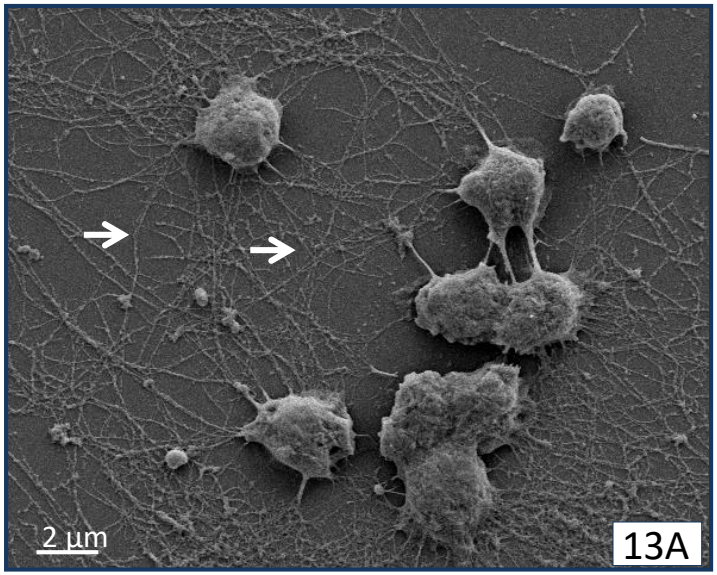
Fig. 9

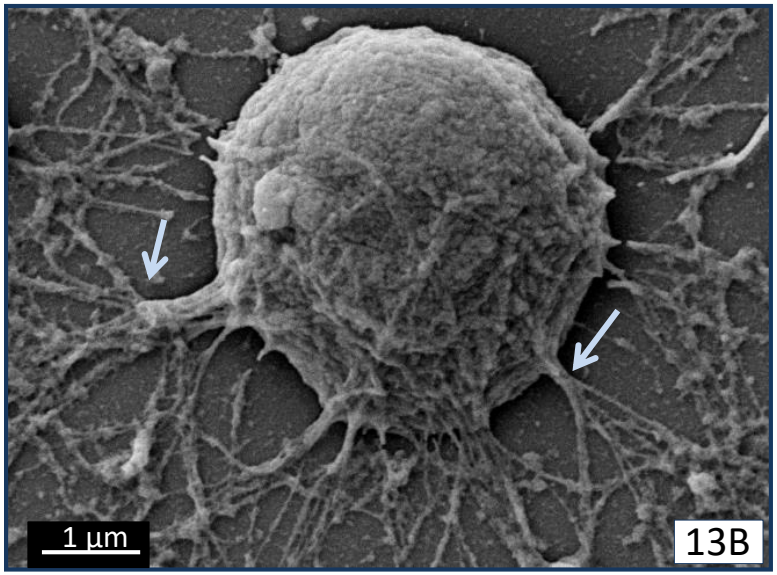

200 µm
10A

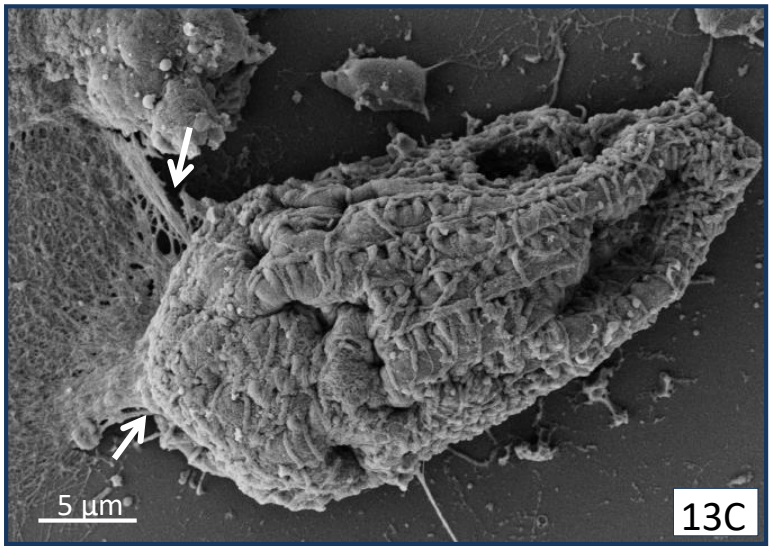

25 µm
10B

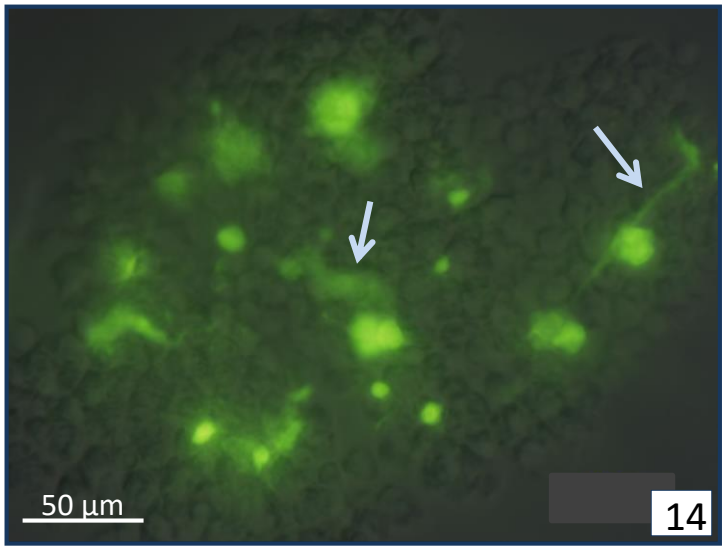

50 µm
11


N
20 µm
12


2 µm
13A


1 µm
13B


5 µm
13C


50 µm
14

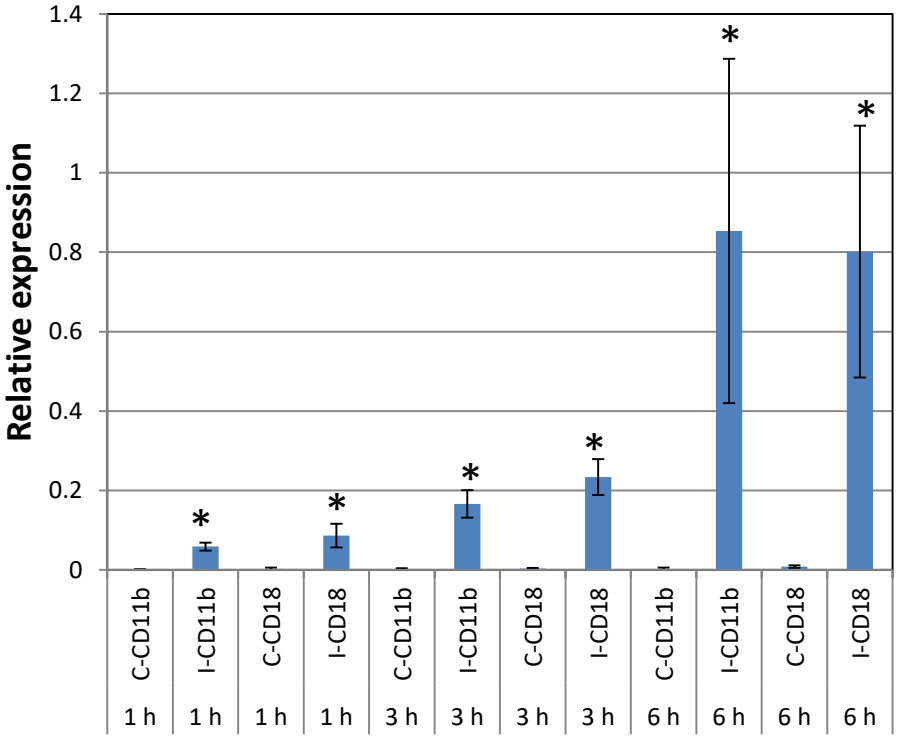
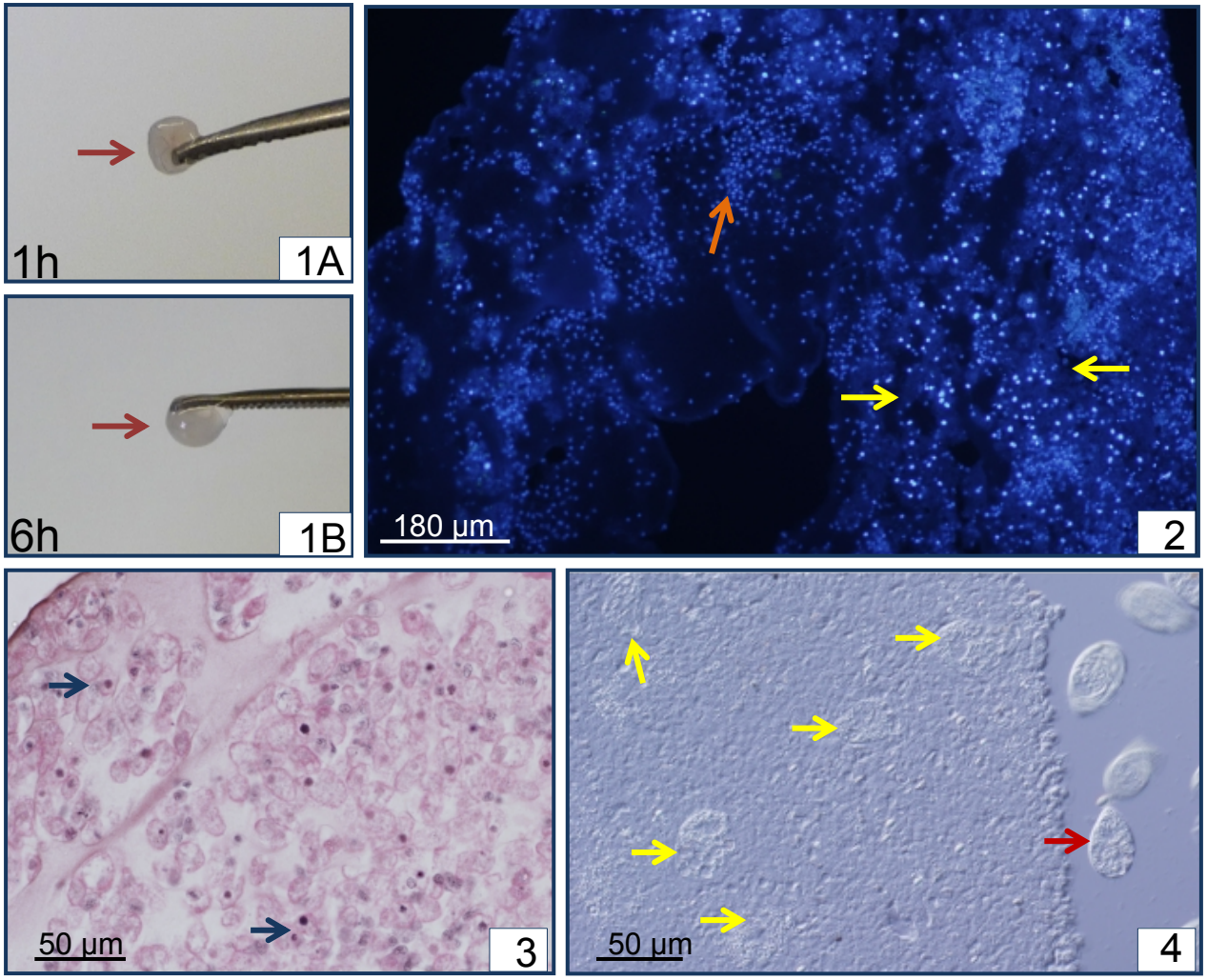
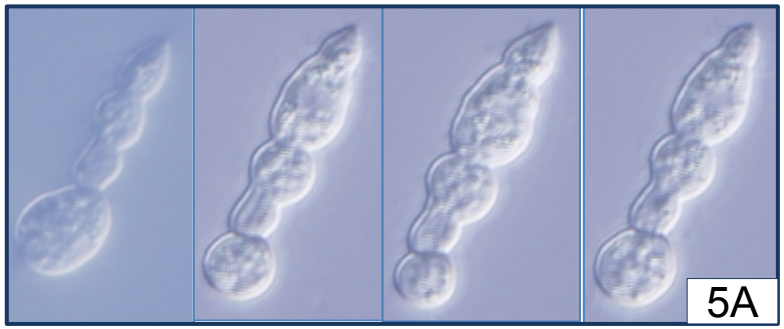
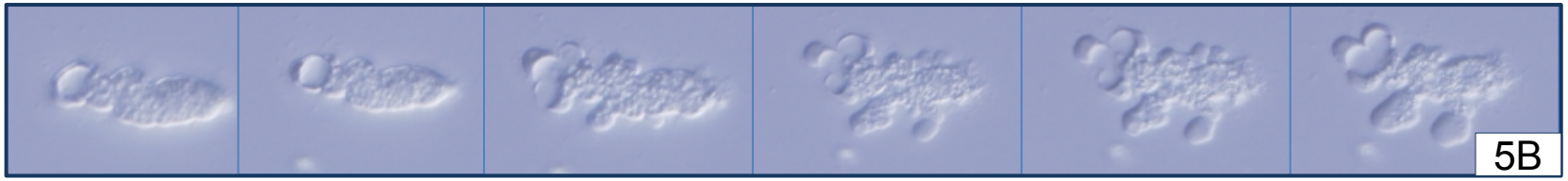
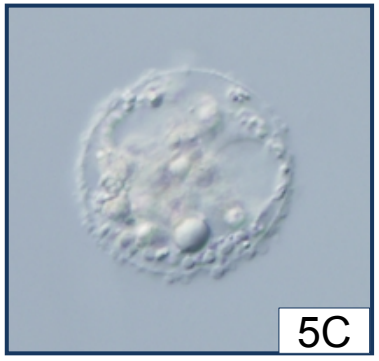

Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C-CD11b 1 h
I-CD11b 1 h
C-CD18 1 h
I-CD18 1 h
C-CD11b 3 h
I-CD11b 3 h
C-CD18 3 h
I-CD18 3 h
C-CD11b 6 h
I-CD11b 6 h
C-CD18 6 h
I-CD18 6 h
*

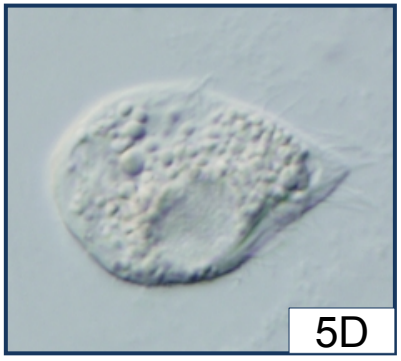
Fig. 15


1h
1A
6h
1B
180 µm
2
50 µm
3
50 µm
4


5A


5B


5C


5D

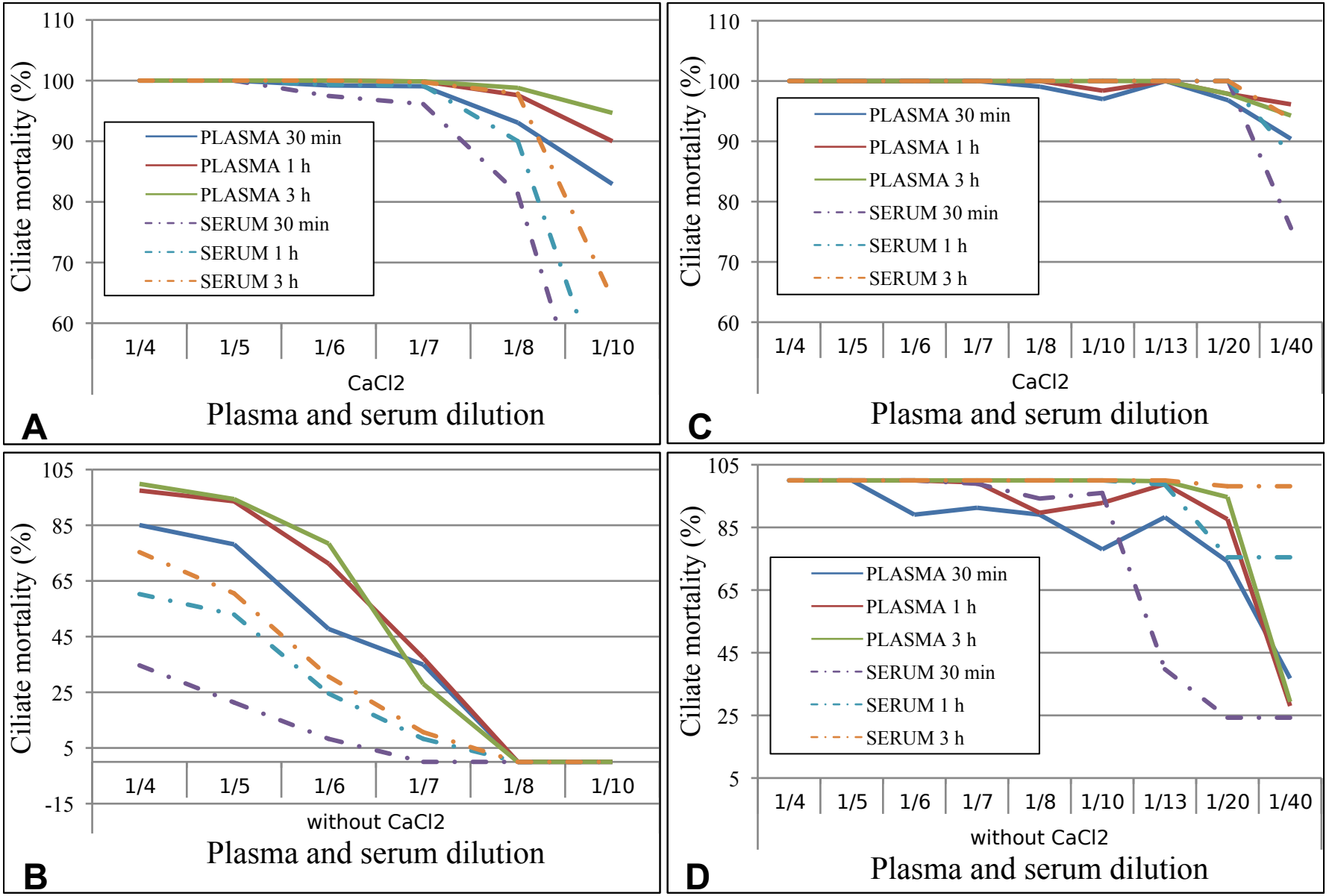

A
Ciliate mortality (%)
110
100
90
80
70
60
PLASMA 30 min
PLASMA 1 h
PLASMA 3 h
SERUM 30 min
SERUM 1 h
SERUM 3 h
1/4
1/5
1/6
1/7
1/8
1/10
CaCl2
Plasma and serum dilution
C
Ciliate mortality (%)
110
100
90
80
70
60
PLASMA 30 min
PLASMA 1 h
PLASMA 3 h
SERUM 30 min
SERUM 1 h
SERUM 3 h
1/4
1/5
1/6
1/7
1/8
1/10
1/13
1/20
1/40
CaCl2
Plasma and serum dilution
B
Ciliate mortality (%)
105
85
65
45
25
5
-15
1/4
1/5
1/6
1/7
1/8
1/10
without CaCl2
Plasma and serum dilution
D
Ciliate mortality (%)
105
85
65
45
25
5
PLASMA 30 min
PLASMA 1 h
PLASMA 3 h
SERUM 30 min
SERUM 1 h
SERUM 3 h
1/4
1/5
1/6
1/7
1/8
1/10
1/13
1/20
1/40
without CaCl2
Plasma and serum dilution

Fig. 6

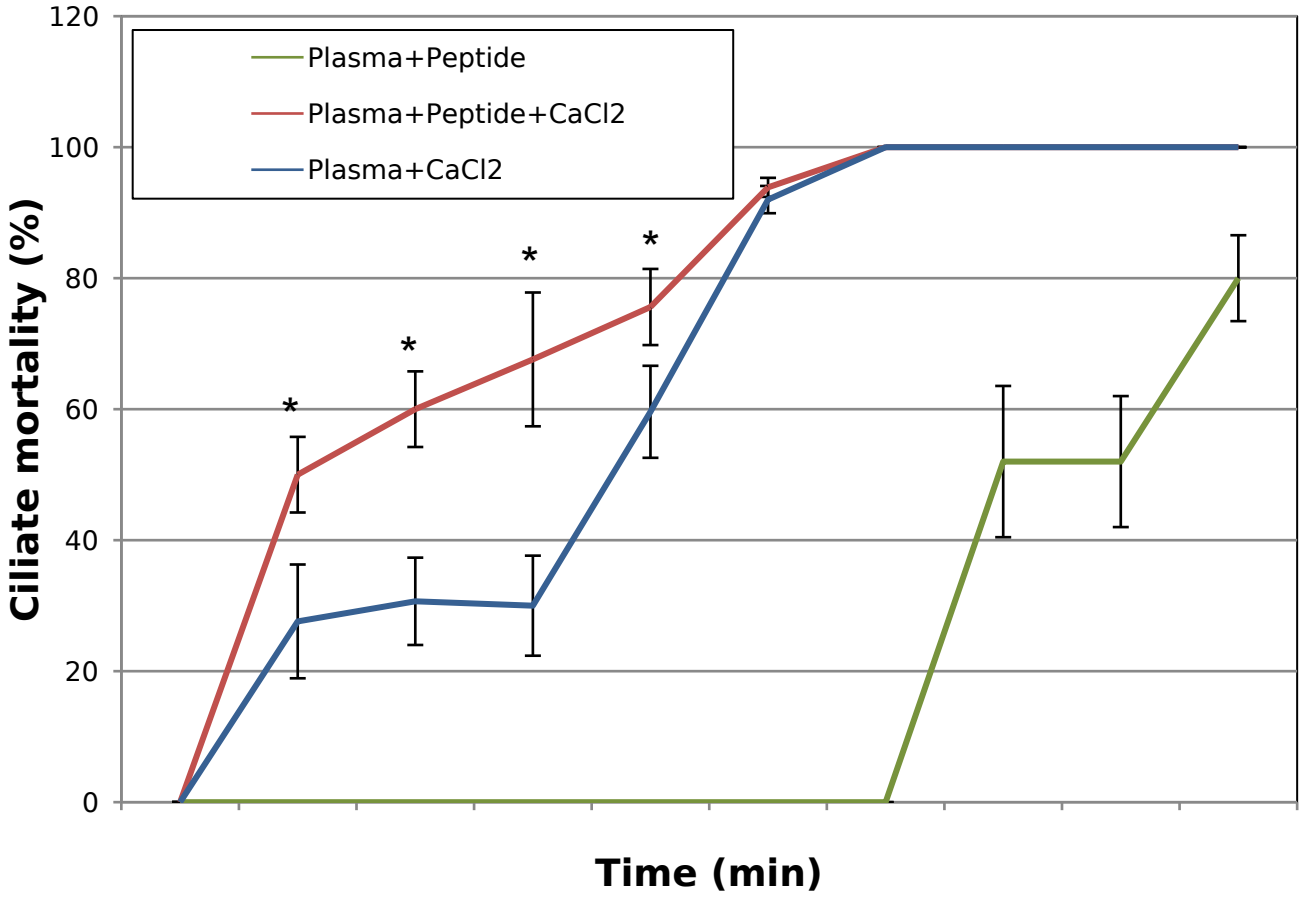
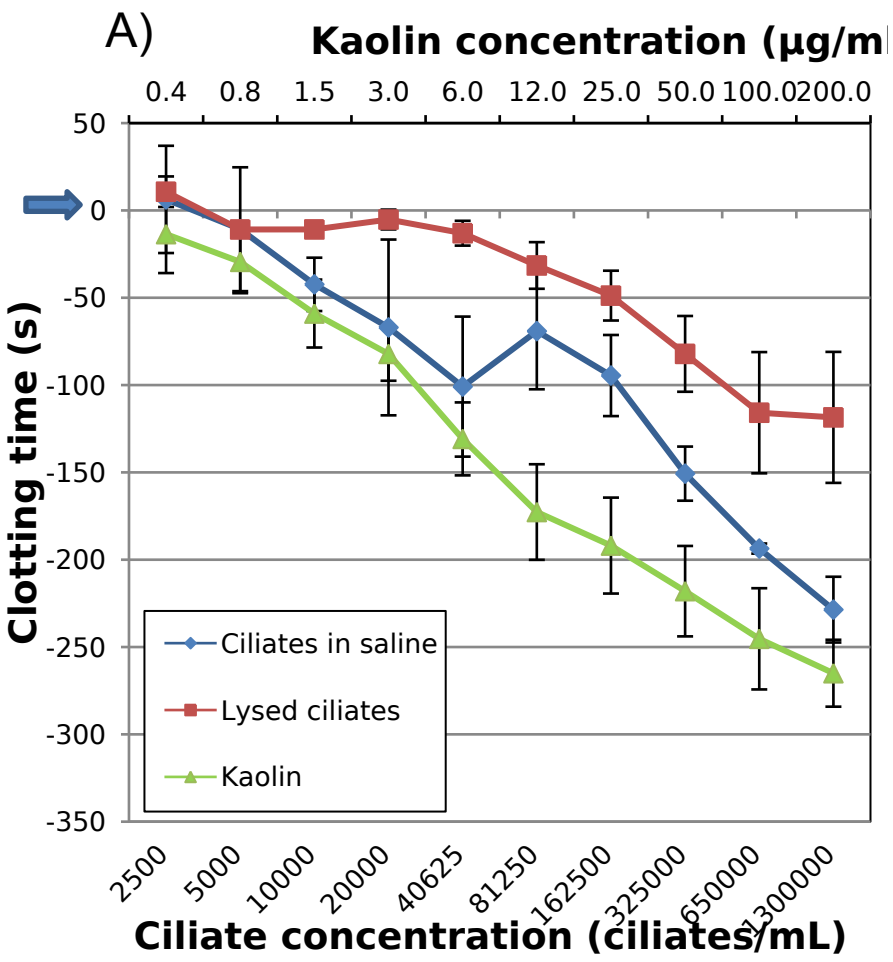

120
100
80
60
40
20
0
Ciliate mortality (%)
Time (min)
Plasma+Peptide
Plasma+Peptide+CaCl2
Plasma+CaCl2
*
*
*
*

Fig. 7


A)
Kaolin concentration (µg/ml
0.4 0.8 1.5 3.0 6.0 12.0 25.0 50.0 100.0 200.0
50
0
-50
-100
-150
-200
-250
-300
-350
Clotting time (s)
Ciliates in saline
Lysed ciliates
Kaolin
2500 5000 10000 20000 40625 81250 162500 325000 650000 1300000
Ciliate concentration (ciliates/mL)

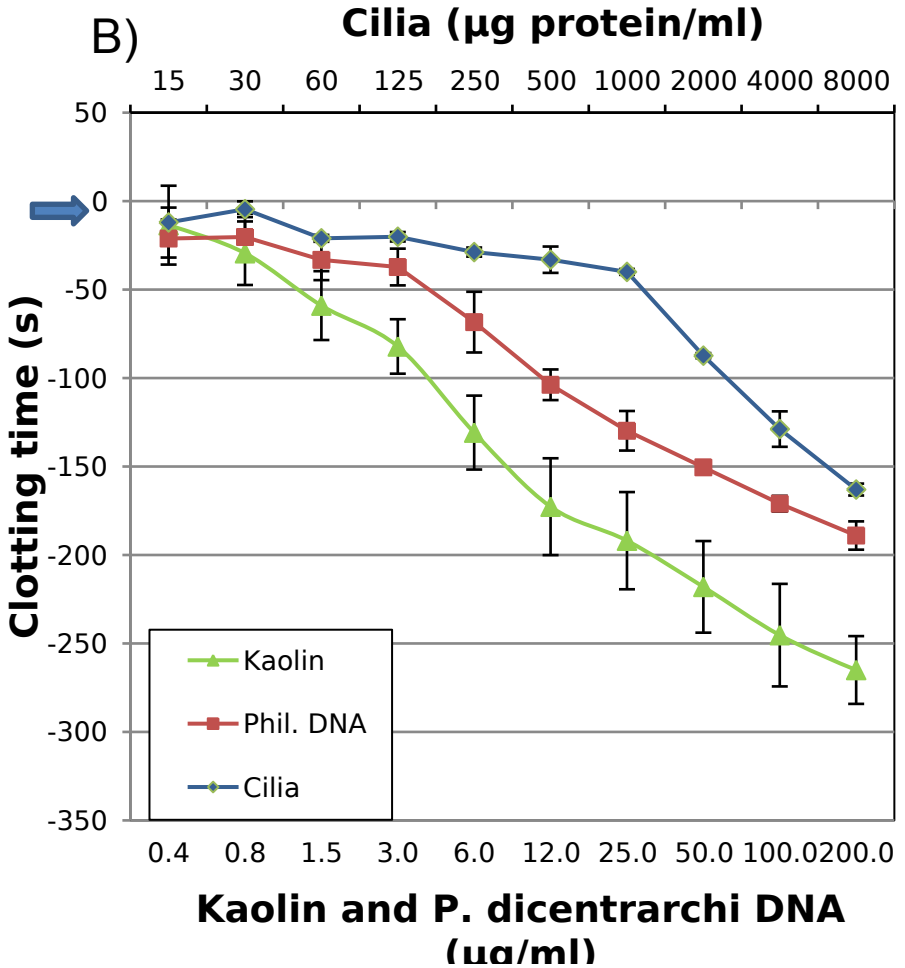

B)
Cilia (µg protein/ml)
15 30 60 125 250 500 1000 2000 4000 8000
50
0
-50
-100
-150
-200
-250
-300
-350
Clotting time (s)
Kaolin
Phil. DNA
Cilia
0.4 0.8 1.5 3.0 6.0 12.0 25.0 50.0 100.0 200.0
Kaolin and P. dicentrarchi DNA (µg/ml)

Fig. 8

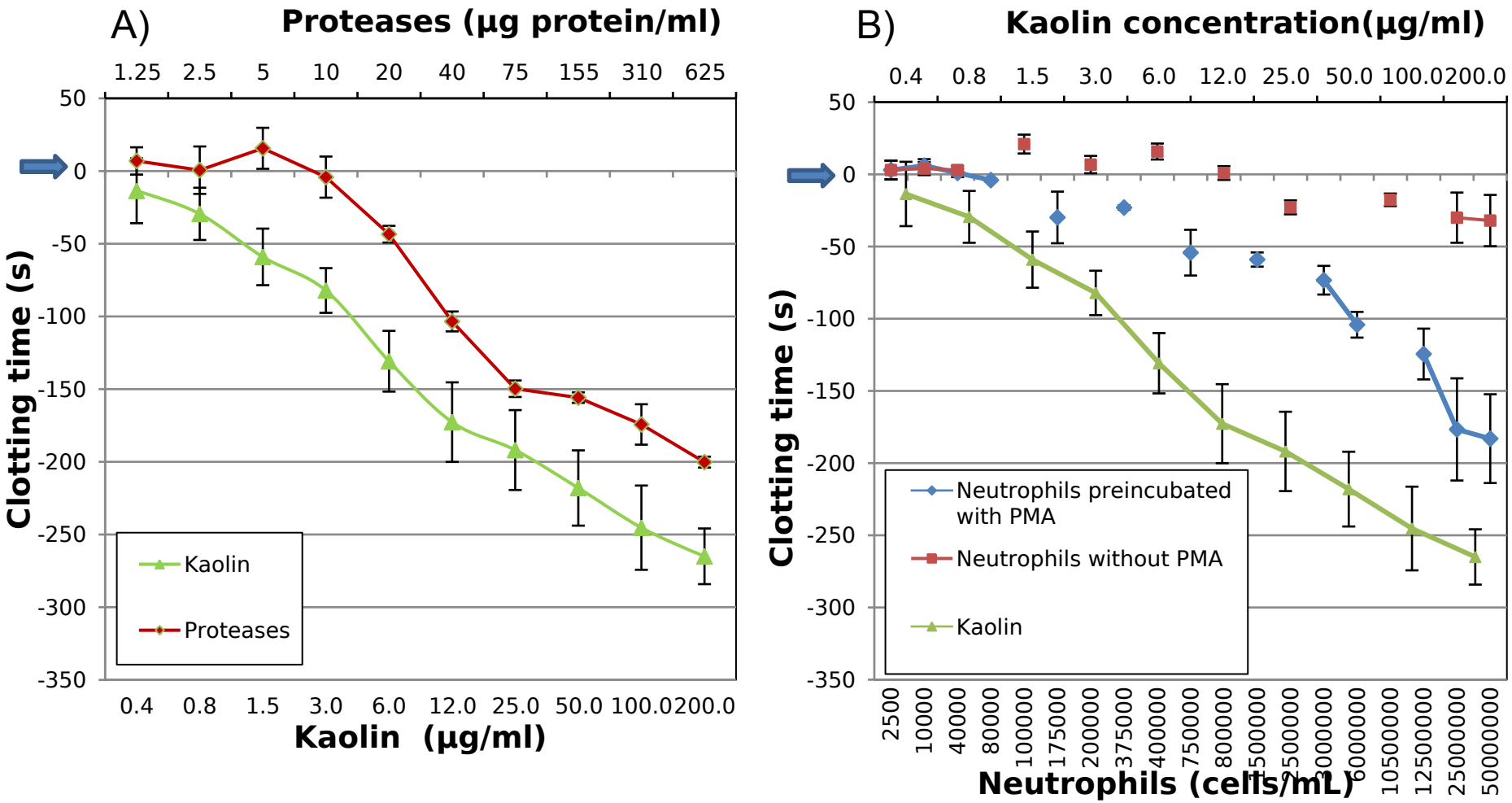
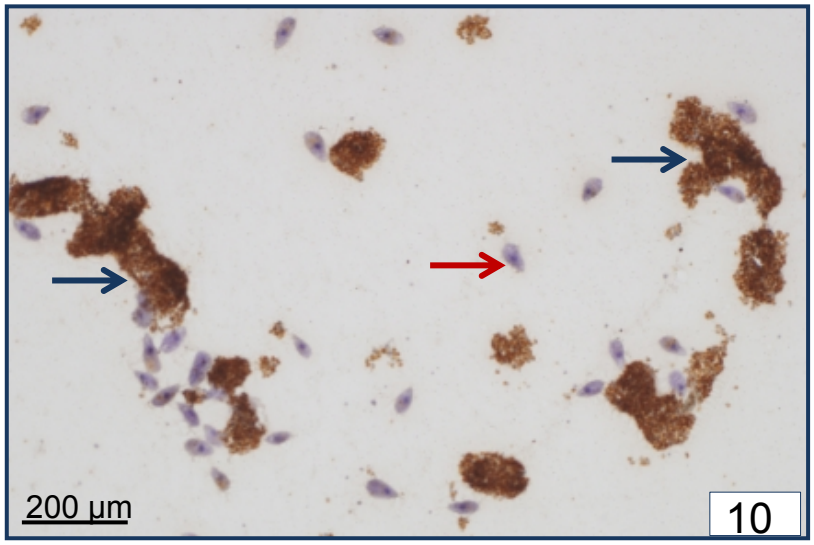
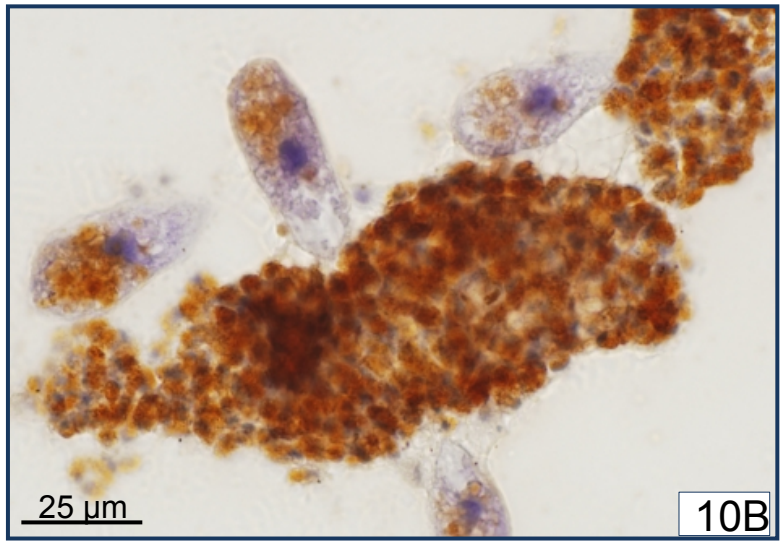
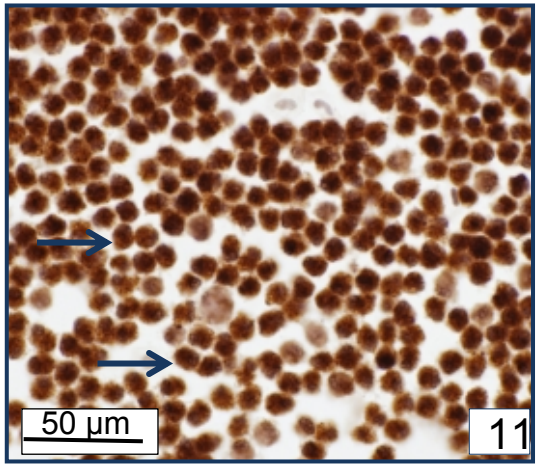
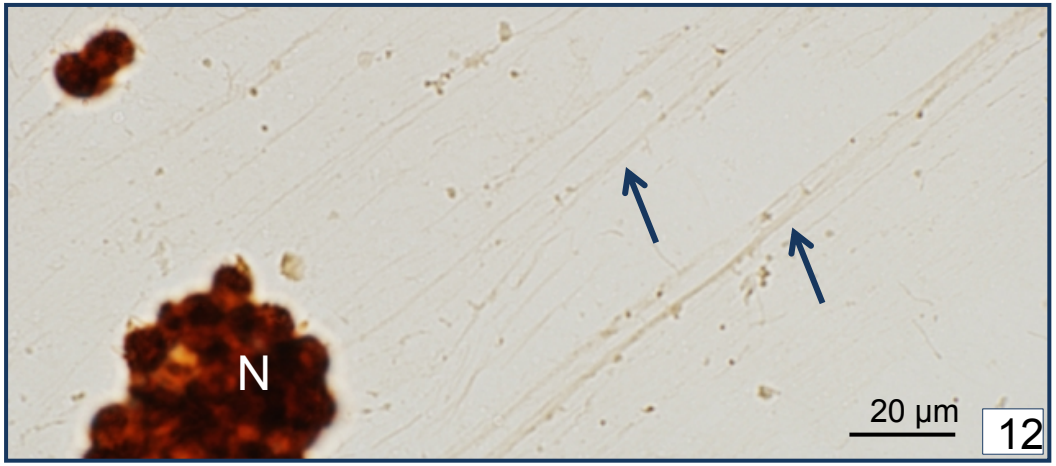

A)
Proteases (µg protein/ml)
1.25 2.5 5 10 20 40 75 155 310 625
50 0 -50 -100 -150 -200 -250 -300 -350
Clotting time (s)
Kaolin
Proteases
0.4 0.8 1.5 3.0 6.0 12.0 25.0 50.0 100.0 200.0
Kaolin (µg/ml)
B)
Kaolin concentration(µg/ml)
0.4 0.8 1.5 3.0 6.0 12.0 25.0 50.0 100.0 200.0
50 0 -50 -100 -150 -200 -250 -300 -350
Clotting time (s)
Neutrophils preincubated with PMA
Neutrophils without PMA
Kaolin
2500 10000 40000 80000 100000 175000 200000 375000 400000 750000 800000 1500000 2500000 3000000 6000000 10500000 12500000 25000000 50000000
Neutrophils (cells/mL)

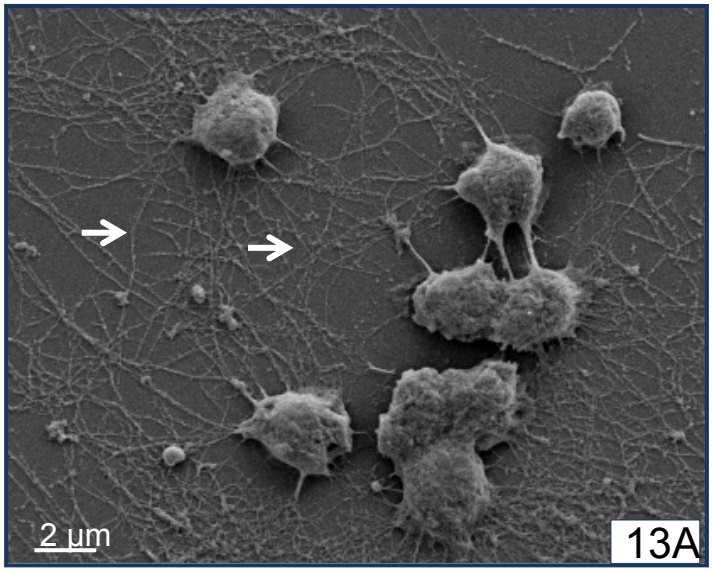
Fig. 9

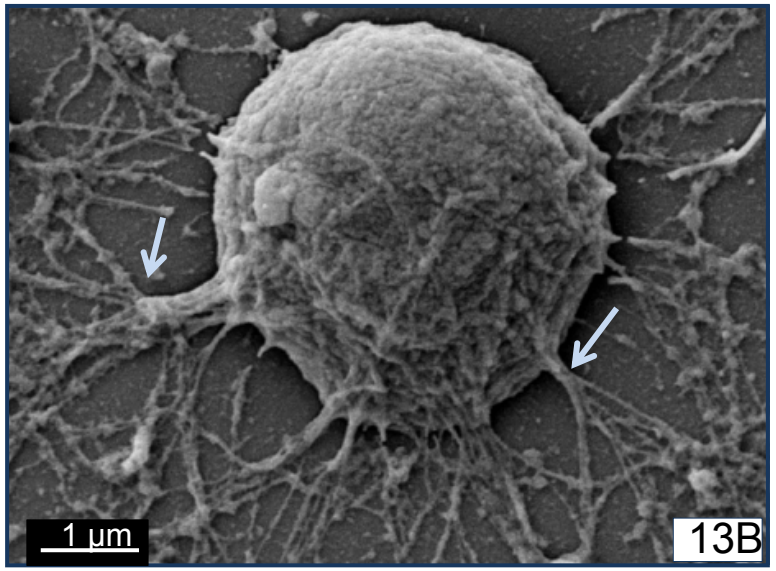

200 µm
10

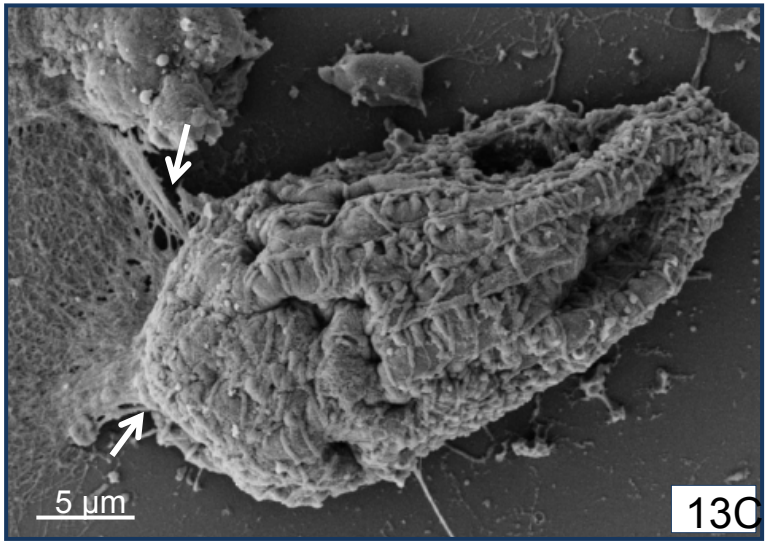

25 µm
10B

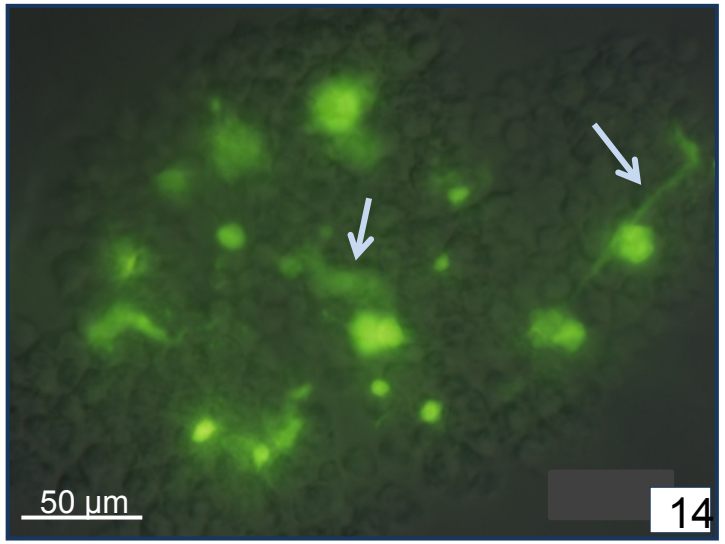

50 µm
11


N
20 µm
12


2 µm
13A


1 µm
13B


5 µm
13C


50 µm
14

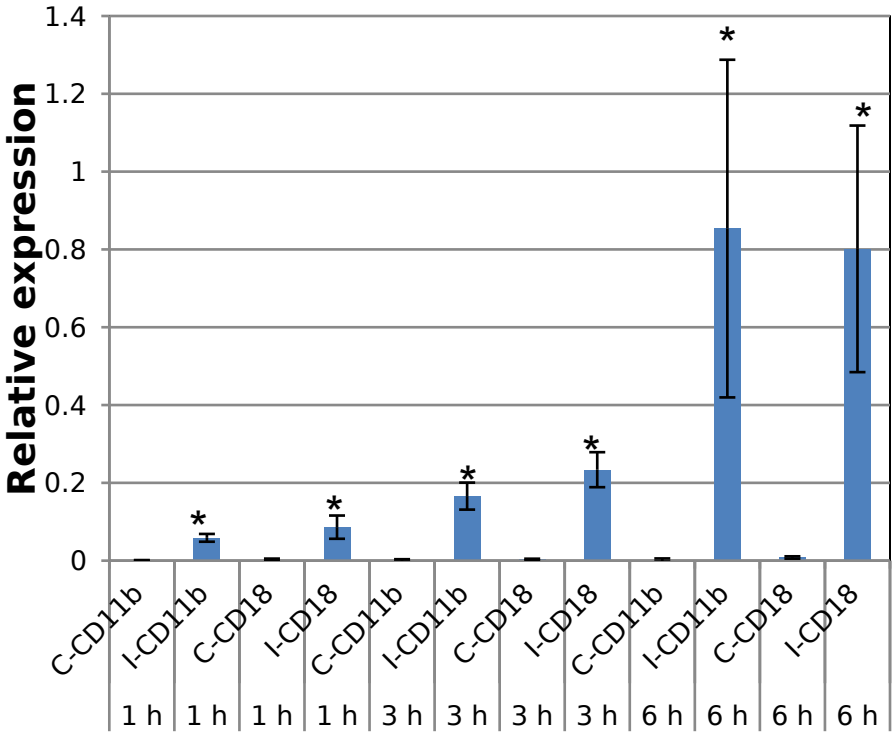

Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C-CD11b
I-CD11b
C-CD18
I-CD18
C-CD11b
I-CD11b
C-CD18
I-CD18
C-CD11b
I-CD11b
C-CD18
I-CD18
1 h
1 h
1 h
1 h
3 h
3 h
3 h
3 h
6 h
6 h
6 h
6 h
*
*
*
*
*
*

Fig. 15