Pollution-induced Immunomodulation in *Biomphalaria glabrata*; Implications for its Relationship with Obligate Parasite *Schistosoma mansoni*

A thesis submitted for the degree of Doctor of Philosophy

By

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Table of Contents

DECLARATION ..... **i**
ABSTRACT ..... **ii**
ACKNOWLEDGMENTS ..... **iii**
LIST OF FIGURES ..... **iv**
LIST OF TABLES ..... **v**
Chapter 1: *Introduction* **1**
1.1: Schistosomiasis 2
1.1.1: Trematodes; parasites of man *and* molluscs 3
1.1.2: Species and distribution of Schistosomes 5
1.1.3: Evolutionary history of *S.mansoni* 8
1.1.4: *Schistosoma* life-cycle 9
1.1.5: Biology of snail stages 11
1.1.5.1: Miracidia; contact and penetration 12
1.1.5.1.1: Epithelial cells 13
1.1.5.1.2: Papilla 13
1.1.5.1.3: Adhesive glands 13
1.1.5.1.4: Penetration gland 13
1.1.5.2: Inside the snail 14
1.1.5.2.1: Development to mother sporocyst 14
1.1.5.2.2: The mother sporocyst 15
1.1.5.2.3: Development of daughter sporocysts 16
1.1.5.2.4: Cercariae; development and release 16
1.2: *Biomphalaria* 17
1.2.1: *Biomphalaria glabrata* 17
1.2.1.1: Evolutionary history and distribution 18
1.2.1.2: BBO2 21
1.2.1.3: Biology 21
1.2.1.3.1: Reproduction 22
1.2.1.3.2: Respiration 23
1.2.2: *Biomphalaria* immune system 25
1.2.2.1: Overview of the invertebrate/molluscan immune system 26
1.2.2.2: Hemocytes of *B.glabrata* 29
1.2.2.3: Interaction with *S.mansoni* 32
1.3: Does pollution affect the *B.glabrata* - *S.mansoni* relationship? 35
1.3.1: Transmission country pollution 37

1.3.1.2: Ecotoxicology; pollutants in the environment 41
1.3.1.2.1: Aquatic ecotoxicology 43
1.3.1.2.2: Immunotoxicology 47
1.3.1.2.2.1: Immunotoxicology in Invertebrates 49
1.3.1.2.2.2: Immunotoxicology in molluscs 50
1.3.1.2.2.3: Immunotoxicology in commercial mollusc species 51
1.3.1.2.2.4: Parasitism and immunotoxicology in molluscs 53
1.3.1.2.2.5: Molluscs and parasites as indicators of ecosystem health and beyond 54
1.4: The hypothesis 56
1.4.1: The importance of method development in testing the hypothesis 56
Chapter 2: *Experimental Organisms; Husbandry and Biology* **60**
2.1: *Biomphalaria* Husbandry 61
2.1.1: Diet 61
2.1.2: Photoperiod 62
2.1.2.1: Photoperiod for infected snails 62
2.1.3: Pests 63
2.1.4: Water 63
2.1.5: Density 64
2.2: *Biomphalaria* cell culture 65
2.2.1: *Biomphalaria* cell buffers 66
2.2.2: Hemolymph collection 67
2.2.2.1: Pooling 69
2.2.2.2: Incubation with test chemicals 72
2.3: Parasite culture 73
2.3.1: Safety considerations 73
2.3.2: Mouse stage and egg collection 74
2.3.3: Egg extraction and hatching 75
2.3.4: Miracidia collection 76
2.3.4.1: Lighting 76
2.3.4.2: Construction of the flask 77
2.3.4.3: Chemical gradient 78
2.3.4.4: Pressure gradient 79
2.3.4.5: Addition of sample 79
2.3.4.6: Miracidia harvesting and transformation 80
2.3.4.7: Findings 80
2.3.5: Miracidia transformation media 81
2.3.6: Infecting snails; background 82
2.3.6.1: Number of miracidia 83

2.3.6.2: Age of snails 83
2.3.6.3: Snail infection protocol 84
2.3.6.4: Shedding 85
2.4: Alternative model: *Marisa cornuarietis* 88
2.4.1: Aim 88
2.4.2: Distribution 89
2.4.3: Biology 90
2.4.3.1: Immunology 91
2.4.4: Use in ecotoxicology 92
2.4.5: Husbandry 94
2.4.5.1: Water temperature 94
2.4.5.2: Light 95
2.4.5.3: Density 96
2.4.5.4: Food 96
Chapter 3: *Assay Validation* **97**
3.1: Test chemicals 98
3.1.1: 17β-estradiol (E2) 98
3.1.2: Bisphenol A (BPA) 99
3.1.3: *p'p*-Dichlorodiphenyldichloroethylene (DDE) 99
3.1.4: Effluent extract 99
3.2: Doses 100
3.3: Statistical Approaches; Methodology and Rationale 101
3.3.1: The dose-response relationship 101
3.3.1.1: How to investigate dose-response relationships 103
3.3.2: Beyond simple linear regression 105
3.3.3: R2 and over-fitting 105
3.3.4: Adjusting variable data 107
3.3.5: Ambiguous instances 108
3.3.6: Where is it acceptable to deviate from the ideals? 108
3.3.7: ANOVA and multiple comparisons 109
3.4: Validation experiments 110
3.4.1: Dose-range effects of test chemicals on hemocyte viability 110
3.4.2: Inhibitor dose-response for *in vitro* assays 112
Chapter 4: *Encapsulations* **114**
4.1: Introduction 115
4.2: Materials and methods 116
4.2.1: Husbandry of test species 116
4.2.2: Fixing of sporocysts 116

4.2.3: Development of chamber slides 118
4.2.4: Surface coating 120
4.2.5: Staining parasites 121
4.2.6: Hemolymph and incubation 121
4.2.7: Analysis; quantifying encapsulation and statistical analysis 122
4.3: Results 124
4.4: Discussion 128
4.5: Conclusion 130
Chapter 5: *Phagocytosis* **131**
5.1: Introduction 132
5.2: Materials and methods 133
5.2.1: Experimental species and husbandry 134
5.2.2: Antigens 134
5.2.2.1: Latex beads 134
5.2.2.2: Bioparticles 135
5.2.2.3: Yeast 135
5.2.3: Exposing hemocytes to antigens; background 136
5.2.3.1: Exposing hemocytes to antigens; my method 136
5.2.4: Imaging flow cytometery 137
5.2.4.1: IFC; pre-acquisition 137
5.2.4.2: IFC; Post-acquisition 137
5.2.5: IFC; masking 139
5.2.6: *In vitro* phagocytosis assays 142
5.2.6.1: *B.glabrata* 143
5.2.6.1.1: Variation in hemocyte phagocytosis rates 143
5.2.6.1.2: *In vitro* exposure of hemocytes to study chemicals 143
5.2.6.1.3: Bioparticle; *in vitro* phagocytosis assay 143
5.2.6.1.4: MTT; *in vitro* phagocytosis assay 143
5.2.6.2: *M.cornuarietis* 146
5.2.6.2.1: Inhibitor dose-response; *in vitro* phagocytosis 146
5.2.6.2.2: Effluent exposure assay; *in vitro* phagocytosis 147
5.2.7: Statistical analysis 147
5.3: Results 148
5.3.1: *B.glabrata; in vitro* phagocytosis assays 148
5.3.1.1: Precision and accuracy 148
5.3.1.2: Bioparticle; *in vitro* phagocytosis assay 149
5.3.1.3: *In vitro* exposure of hemocytes to study chemicals 150
5.3.2: *M.cornuarietis* 153

5.3.2.1: Inhibitor dose-response; *in vitro* phagocytosis 156
5.3.2.2: Effluent exposure assay; *in vitro* phagocytosis 157
5.3.2.3: MTT; *in vitro* phagocytosis assay 158
5.4: Discussion 159
Chapter 6: *Cell Motility*; *developing of a low-cost assay with wider applications* **167**
6.1: Introduction 168
6.2: Materials and methods 170
6.2.1: Construction and Calibration of the microscope system 170
6.2.1.2: Microscopes 171
6.2.1.3: Lighting 171
6.2.1.4: Heating 171
6.2.1.5: Resolution 172
6.2.1.6: Magnification and Horizontal field of view 172
6.2.2: Software 173
6.2.2.1: Image capture 173
6.2.2.2: Image preparation and analysis 174
6.2.3: Assays 175
6.2.3.1: Cell tracking 175
6.2.3.2: Scratch assay 176
6.2.3.3: *Artemia* development 176
6.2.3.4: Test chemical assays 176
6.2.4: Statistical analysis 177
6.3: Results 177
6.3.1: Microscope Functional Parameters 177
6.3.1.1: Heating stability 177
6.3.1.2: Field of view and magnification 178
6.3.1.3: Pixel resolution 181
6.3.1.4: Spatial resolution 182
6.3.1.5: Comparison with a conventional microscope 182
6.3.1.6: Lighting 182
6.3.2: Assays and applications 183
6.3.2.1: Cell velocity 183
6.3.2.2: Scratch Assay 184
6.3.2.3: *Artemia* development 185
6.3.2.4: Study chemical testing 186
6.4: Discussion 187
Chapter 7: *In vivo* Effluent-Exposure Study **193**
7.1: Introduction 194

7.1.1: Aims 196
7.2: Materials and Methods 197
7.2.1: Exposure Methods 197
7.2.2: Pilot Study to Determine Optimal Exposure Conditions 198
7.2.3: Primary Study 201
7.2.3.1: Exposure of *B. glabrata* to an effluent extract 202
7.2.3.1.1: Effluent Collection and Stock Preparation 202
7.2.3.1.2: Solid phase extraction; disk conditioning and effluent extraction 203
7.2.3.1.3: Solid phase extraction; disk elution and stock creation 204
7.2.3.1.4: Chemical Composition of Samples 206
7.2.4: Exposure Setup 206
7.2.5: Experimental Endpoints 208
7.2.5.1: Histology 209
7.2.6: Statistical Analysis 210
7.3: Results 210
7.3.1: Pilot Study 211
7.3.2: Primary Study 212
7.3.2.1: Size; post-exposure, pre-infection 212
7.3.2.2: Mortality 213
7.3.2.3: Shedding 214
7.3.2.4: Motility 215
7.3.2.5: MTT 216
7.3.2.6: Histology 217
7.4: Discussion 228
Chapter 8: *General Discussion* **236**
8.1: Conclusion 243
REFERENCES **244**

DECLARATION

The work submitted in this thesis was conducted between 2011 and 2014 at Brunel University London. This work was carried out independently and has not been submitted for any other degree.

Abstract

Aquatic pollution from urban and industrial effluents represents a growing area of concern. The number and volume of xenobiotic chemicals in aquatic ecosystems is alarmingly high, due in part to increasing globalization and the associated demands. Invertebrates, in particular molluscs, represent species of great commercial importance and can therefore fail to be considered in terms of their significance in the transmission of human disease. *Schistosoma mansoni* is a trematode parasite transmitted to humans by aquatic snails of the genus *Biomphalaria*. *S.mansoni* infects up to 200 million people globally and transmission primarily occurs in developing countries with poor infrastructure, factors which also happen to be associated with high levels of aquatic pollution. Despite the medical importance of *S.mansoni* and its occurrence in potentially polluted environments, very few attempts have been made to study this parasite-host relationship in the context of ecotoxicology. In this thesis I have applied both adapted and novel approaches in order to combine the fields of parasitology and ecotoxicology toward a better understanding of the effects of globally-prevalent xenobiotic chemicals on the *S.mansoni-B.glabrata* relationship. *In vitro* assays, with various end-points, were performed based on exposure of hemocytes, the primary immune effector cells of molluscs, while whole snails were developmentally exposed to an effluent extract and subsequently infected as part of an *in vivo* study. Taken together, my results suggest that the immunocompetance of *B.glabrata* hemocytes is broadly reduced in the presence of DDE, BPA, E2 and an effluent extract; chemicals that occur at high levels in transmission countries. Reduction in the key hemocyte functions of motility, phagocytosis and encapsulation, caused by exposure to these chemicals, appears to be exacerbated by subsequent *S.mansoni* infection which results in an opportunity for increased parasite shedding. My hope is that this broad work will serve as a reference and facilitate more focused studies, particularly of a molecular and epidemiological nature, into what is an understudied and potentially very important topic with the potential for human health implications.

List of Figures

Figure 1.1: Representation of the typical life-cycle stages of animals in the class *Trematoda* **4**

Figure 1.2: Global distribution of the five human-infective *Schistosoma* species of medical significance **7**

Figure 1.3: The life cycle of the three most prevalent human-infective *Schistosoma* species; *S.mansoni, S.haematobium* and *S.japonicum* **10**

Figure 1.4: A closer look at the physiology of the Intra-molluscan stages of *S.mansoni* development **12**

Figure 1.5: Phylogenetic relationships within and between the African and American species of the genus *Biomphalaria.* **19**

Figure 1.6: Geographic distribution of the phylogenetic relationships among the five clades of the species *Biomphalaria glabrata.* **20**

Figure 1.7: Anatomical diagram showing the basic body plan of *B.glabrata* **22**

Figure 1.8: Anatomical diagram of the respiratory process of *B.glabrata* and the proposed role of water-flow. **24**

Figure 1.9: A comparison between the respiratory pigments of snails from the genus *Biomphalaria* and those from another, more typical, gastropod. **25**

Figure 1.10: Comparison between the two main functional states in which immunocompetent hemocytes of *Biomphalaria glabrata* are typically observed *in vitro.* **30**

Figure 1.11: Pollutant classes and levels present in freshwater bodies of *S.mansoni* transmission countries **39**

Figure 1.12: Pollutant classes and levels present in aquatic molluscs from *S.mansoni* transmission countries **40**

Figure 1.13: Representation of the effect that the focus of a study has on its level of mechanistic/ecological relevance **59**

Figure 2.1: Photograph of *B.glabrata* hemocytes attacking and encapsulating a piece of foreign debris. **69**

Figure 2.2: Diagrammatic example of the process by which my technical and experimental units were generated **72**

Figure 2.3: Photographs of my miracidia collection device. **77**

Figure 2.4: Demonstration of the chemical gradient formed between the miracidia collection chamber and the main flask **79**

Figure 2.5: Miracidia developing into mother sporocysts in DMEM media **82**

Figure 2.6: Photograph of pipette tip demonstrating my method for catching precise numbers of miracidia **85**

Figure 2.7: Photograph of my method for cercariae quantification **87**

Figure 2.8: The global distribution of endemic and invasive *M.cornuarietis* **90**

Figure 2.9: Illustration of *Marisa cornuarietis.* **91**

Figure 3.1: Hypothetical examples of strong linear regression relationships seen in dose-response assays **104**

Figure 3.2: Over-fitting demonstrated by the best-fit regression model for a hypothetical data set **106**

Figure 3.3: Dose-response assays used to determine whether significant toxicity occurred to hemocytes across my chosen dose range for each test chemical **111**

Figure 3.4: Dose-response relationships between the metabolic inhibitor NaN_3 and different endpoints of hemocyte health **113**

Figure 4.1: Development process for my purpose-built chamber-slide for use in the *in vitro* encapsulation assay **119**

Figure 4.2: Photographic examples of sporocyst encapsulation index from two different studies. **123**

Figure 4.3: Example of fluorescence microscopy used in the scoring index of my encapsulation assays. **124**

Figure 4.4: Regression relationships between log dose (X axis) of test chemicals against encapsulation response toward *S. mansoni* sporocysts by *B. glabrata* hemocytes exposed *in vitro* (Y axis) **126**

Figure 4.5: Dunnett's multiple comparisons between mean value of hemocyte encapsulation index (X) at each test chemical and dose (Y) **127**

Figure 5.1: Identifying properly focused hemocytes using a frequency histogram generated with ImageStream gradient Root Mean Squared (RMS) data. **138**

Figure 5.2: Identifying single cells using a scatter-plot based on ImageStream aspect ratio and area data from the 'focused' hemocyte population **138**

Figure 5.3: Identifying hemocyte images containing a fluorescent latex bead using a frequency histogram generated with fluorescent intensity data based on the 'single cell' population **139**

Figure 5.4: Example of the process of developing a custom mask in order to restrict the analysis to the cell image only and exclude external objects **140**

Figure 5.5: Frequency histogram based on the bead+ population gated into regions of images containing internalization positive (+) and negative (-) cells, according to the custom mask **141**

Figure 5.6: Example of a situation in which the custom mask correctly identifies an internalized bead while excluding a non-internalized one **141**

Figure 5.7: Frequency histogram based on the internalization positive population showing the quantity of hemocytes which contain specific numbers of beads **142**

Figure 5.8: Example images of the setup for the MTT assay, adapted for studying phagocytosis in *B.glabrata*..... **146**

Figure 5.9: Frequency histograms of fluorescent intensity from Bioparticles exposed to hemocytes and incubated with or without phagocytosis inhibitor cytochalasin B..... **149**

Figure 5.10: Level of phagocytosis as represented by the percentage of fluorescence positive cells from hemocytes incubated with or without the inhibitor cytochalasin B **150**

Figure 5.11: Regression relationships between log dose (X axis) of test chemicals against phagocytosis of latex beads by *B. glabrata* hemocytes exposed *in vitro* (Y). **151**

Figure 5.12: Multiple comparisons between mean number of beads per cell (X) at each test chemical and dose (Y) **152**

Figure 5.13: Scatter graph showing phagocytosis of yeast by *M.cornuarietis* hemocytes and the ability to separate out yeast clumps based on fluorescent profile **153**

Figure 5.14: Frequency histogram of the number of yeast cells inside phagocytosis positive *M.cornuarietis* hemocytes according to the spot count mask **156**

Figure 5.15: Regression relationship between phagocytosis response as measured by mean number of yeast per *Marisa* hemocyte (Y axis) and the percentage of NaN_3 to which it was exposed (X axis). **157**

Figure 5.16: Regression relationships between log dose (X axis) of effluent extract against phagocytosis of yeast by *Marisa* hemocytes exposed *in vitro* (Y)..... **158**

Figure 6.1: Different images of the microscope system **170**

Figure 6.2: Screenshot of the Autokams software interface **174**

Figure 6.3: Raw image preparation and analysis process in ImageJ **175**

Figure 6.4: Incubator temperature stability over time **178**

Figure 6.5: Images used for calibration of the microscopes **179**

Figure 6.6: Comparison of MDA-MB-231 cell images between my system and a conventional optical microscope **180**

Figure 6.7: Comparison of digital microscope pixel resolutions between 640x480 and 1280x960 ..... **181**

Figure 6.8: Screenshot of the Autokams interface showing the result of illumination from the in-built LEDs alone. **183**

Figure 6.9: Mean velocity (± SE) in µm/min of the 4 cell types **184**

Figure 6.10: Screenshot of Autokams interface showing individual scratch assays simultaneously recorded on each camera **185**

Figure 6.11: Screenshot of Autokams which demonstrates the ability of the microscope system to image distinct samples in different manners simultaneously **186**

Figure 6.12: Regression relationships between log dose (X axis) of test chemicals against *B. glabrata* hemocyte motility exposed *in vitro* (Y) **187**

Figure 7.1: Photograph of pilot experiment setup with beakers grouped according to volume (but not water change frequency) **200**

Figure 7.2: The process of priming and conditioning a C18 disk and extracting an effluent sample through it **204**

Figure 7.3: The process of eluting the bound effluent chemicals from the C18 SPE disk. **205**

Figure 7.4: Photographs of the effluent extraction process **206**

Figure 7.5: Photograph of the main study setup for adult snail exposure to the effluent extract **207**

Figure 7.6: Experimental setup for infected (and uninfected) snails after the period of effluent exposure **208**

Figure 7.7: Linear regression model of the relationship between snail density and size **211**

Figure 7.8: Linear regression analysis of the relationship between log effluent extract concentration and snail shell length (mm). **213**

Figure 7.9: Linear regression model of the relationship between log effluent concentration and the mean number of cercariae shed per snail. **214**

Figure 7.10: Visual representation of the multiple regression model built to predict *B.glabrata* hemocyte motility (mean velocity µm/min) based on the snails' *S.mansoni* infection status (positive or negative) and effluent concentration (0, 0.1, 1, 10x - $\log_{10}$).. ..... **215**

Figure 7.11: Visual representation of the multiple regression model built to predict *B.glabrata* hemocyte phagocytosis ability (measured as mean absorbance at 570nm) based on the snails' *S.mansoni* infection status (positive or negative) and effluent concentration (0, 0.1, 1, 10x - $\log_{10}$)..... **217**

Figure 7.12: Histology images from snail 1, 0x **218**

Figure 7.13: Histology images from snail 2, 0.1x **218**

Figure 7.14: Histology images from snail 3, 1x **219**

Figure 7.15: Histology images from snail 4, 10x **220**

Figure 7.16: Histology images from snail 5, 0x **220**

Figure 7.17: Histology images from snail 6, 0.1x **221**

Figure 7.18: Histology images from snail 7, 1x **221**

Figure 7.19: Histology images from snail 8, 10x **222**

Figure 7.20: Comparison of digestive gland sections from snail 5 (0x) and snail 8 (10x) showing the extent of toxic damage **223**

Figure 7.21: Histology images from snail 9, 0x **224**

Figure 7.22: Histology images from snail 10, 0.1x **224**

Figure 7.23: Histology images from snail 11, 1x **225**

Figure 7.24: Histology images from snail 12, 10x **226**

List of Tables

Table 1.1: Medically important human-infective species of the genus *Schistosoma* **6**

Table 1.2: An example of some of the different populations and compositions of *B.glabrata* hemocytes reported in the scientific literature **31**

Table 1.3: An example of selected studies which focused on the effects of various xenobiotics on the outcome of infectious diseases in different aquatic species. **48**

Table 1.4: Compilation of various studies on the immunotoxic effects of different pollutants on commercial mollusc species **52**

Table 1.5: A meta-analysis of reported associations between anthropomorphic environmental impacts and parasite abundance. **55**

Table 2.1: Composition of buffers used in *B.glabrata* hemocyte *in vitro* culture and manipulation **67**

Table 2.2: Relationship between snail water temperature and mean number of days to first cercarial shedding **86**

Table 3.1: Studies which have reported some of the high-levels of my chosen test-chemicals in freshwater-bodies of *S.mansoni* transmission countries **101**

Table 3.2: A hypothetical example to demonstrate the difference in adjusted R^2 values when analysing individual data points or mean values for Y **107**

Table 4.1: Some examples of semi-quantitative scoring definitions of encapsulation index from various studies. **122**

Table 5.1: Proposed interaction between a test chemical and hemocyte function and the expected outcome this would indicate from the MTT assay **145**

Table 5.2: Performance of the custom mask based on observed (result from custom mask) vs expected (result from visual observation) values for phagocytosis classification **149**

Table 5.3: Criteria used for differentiation of objects based on their different Channel 2 and Channel 5 intensities **154**

Table 7.1: Example of the calculations for the total volume of final effluent required in order to obtain the desired volume of extract for use in the *in vivo* study. **199**

Table 7.2: Experimental groups for the pilot study based on density and water change frequency **200**

Table 7.3: Examples of different variables from studies which have exposed *B. glabrata in vivo* under both static and static-renewal conditions **201**

Table 7.4: Summary of mean size (mm), weight (g) and % mortality according to snail density (water volume in ml) and water change frequency **212**

Table 7.5: Summary statistics from multiple regression analysis model built to predict mortality, based on the snails' *S.mansoni* infection status (positive or negative) and the concentration of effluent extract (0, 0.1, 1, 10x - $\log_{10}$) to which it was exposed *in vivo*. **214**

Table 7.6: Summary statistics from multiple regression analysis model built to predict motility (measured as mean velocity µm/min) of hemocyte cells taken from *B.glabrata*, based on the snails' *S.mansoni* infection status (positive or negative) and the concentration of effluent extract (0, 0.1, 1, 10x - $\log_{10}$) to which it was exposed *in vivo*. **215**

Table 7.7: Summary statistics from multiple regression analysis model built to predict phagocytosis (measured as mean absorbance) ability of hemocyte cells taken from *B.glabrata*, based on the snails' *S.mansoni* infection status (positive or negative) and the concentration of effluent extract (0, 0.1, 1, 10x - $\log_{10}$) to which it was exposed *in vivo*. **216**

Table 7.8: Summary notes for each sectioned snail based on histological evaluation **227**

Table 7.9: Number of daughter sporocysts, cercariae and a combined total number of parasites for each snail/dose. **228**

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Chapter 1: *Introduction*

1.1. Schistosomiasis

Schistosomiasis, also known as bilharzia, is a term used to refer to human infection by any of five major species of trematode worm belonging to the genus *schistosoma*. It is a tropical disease endemic to the developing world and is considered the second most important human parasitic disease after malaria, with more than 200 million people infected across 78 countries. This figure refers to those with active/current schistosome infections while a similar number are thought to be in a post-infection stage and continue to have residual morbidity, which therefore would bring the global number of people affected by some form of schistosomiasis closer to 400 million (Colley *et al*., 2014). Pathology associated with the parasite is estimated to cause up to 200,000 deaths every year and cost economies between 1.7 and 4.5 million disability-adjusted life years (DALYs), a measurement which takes into account lost productivity rather than just mortality (Baeza Garcia *et al*., 2010; Mohamed *et al*., 2012).

Despite the seriousness of the disease, schistosomiasis is still considered to be a 'neglected tropical disease', a disease which occurs in low-income regions and receives significantly less research and treatment than comparatively less serious diseases occurring in developed countries. Reasons for neglect are not certain, but are possibly due to the lack of public awareness since these diseases tend to have long incubation periods and are usually chronic conditions rather than acute. Schistosomiasis can cause acute illness, but its main public health impact is due to chronic infections which lead to increased risk of anaemia, growth stunting and cognitive impairment in children and also the exacerbation of co-infections like HIV. Depending on the species, the liver, intestine, spleen, lungs and urogenital system are affected causing serious health problems, most notably bladder cancer, in later life (Knopp *et al*., 2012).

There is currently no vaccine against schistosomiasis and in the past 10 years resistance to the main anti-schistosome drug 'Praziquantel' has been observed, while very few potential replacements have emerged (Bayne, 2009). Therefore, it seems that schistosomiasis will continue to be a serious health problem in certain parts of the world while drug and vaccine research continues and new approaches to tackle the disease are developed.

Clearly, a detailed understanding of the organism which causes this costly disease, and those which transmit it, is crucial in any efforts to combat it. This is made more important by the complex and intimate interactions that this trematode parasite has with its hosts, both human, and equally importantly but often over-looked, non-human.

1.1.1. Trematodes; parasites of man *and* molluscs

Trematodes, commonly referred to as flukes, are a class within the 'flatworm' phylum of Platyhelminthes to which the genus *Schistosoma* belongs. Trematodes are typically divided into two sub-classes based on reproductive strategy; the *Aspidogastrea* and the much larger sub-class, the *Digenea*.

There are approximately 18,000 nominal species of digenic trematode, nearly all of which are primary parasites of molluscs and most of which are obligate gastropod parasites. Digeans typically exhibit considerable host specificity. In a coevolutionary context there are no parasites which are so inextricably linked to a single group of hosts as digenetic trematodes are to snails and other molluscs (Esch *et al*., 2001; Loker, 2010).

The trematode undergoes a complex developmental process within the mollusc/primary host, including extensive proliferation *via* several asexual cycles (Figure 1.1) typically lasting several weeks, to eventually produce embryos that release large numbers of the next free-living stage, the cercariae, which are directly infective to the secondary host (Loker, 2010). The free-living larval stages of Digeneans show a variety of behavioural adaptations which help them to locate and invade their secondary hosts. After responding to environmental cues during the phases of dispersal and microhabitat-selection, they often use host-chemical signals for short-range orientation, identification of the host species and for invasion and transformation processes (Haberl *et al*., 2000). Sexually mature adult worms live in a vertebrate host and, in the case of diecious species they mate, then release eggs into the aquatic environment. The first free-living larval stage, the miracidia, hatches from the egg then locates and infects a compatible mollusc and the life cycle continues (Morley, 2010).

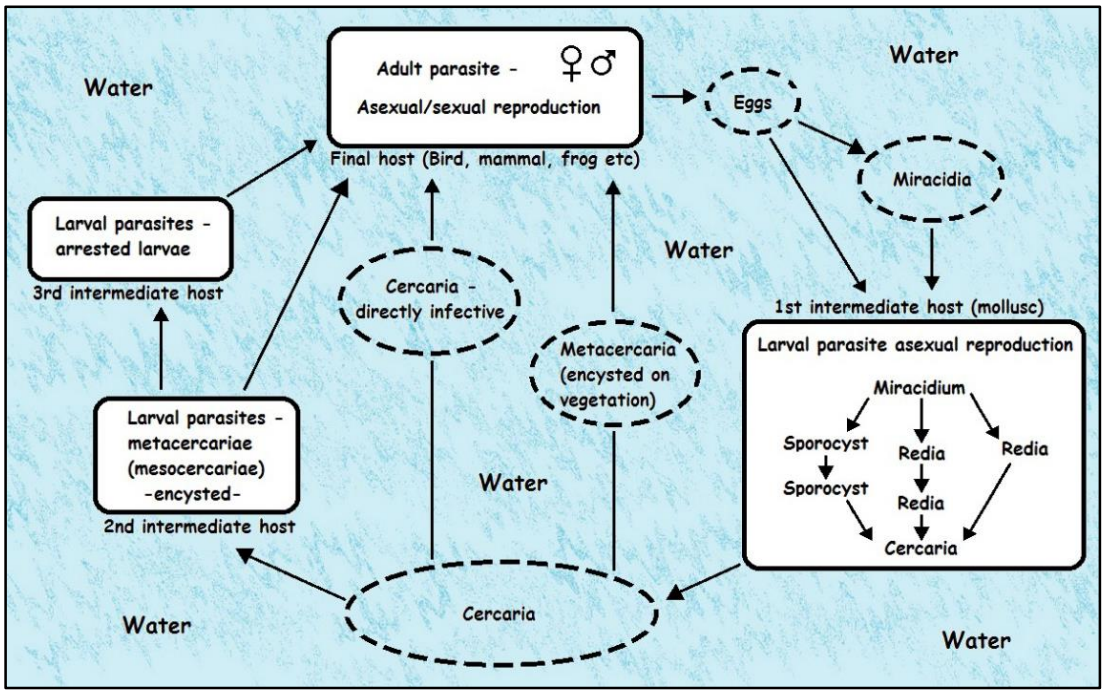


Figure 1.1. Representation of the typical life-cycle stages of animals in the class Trematoda. *Shaded blue background represents the aquatic environment; dashed ovals represent trematode stages which are present in water and independent of a host; solid white boxes represent an animal host; arrows indicate the direction of the life cycle progression.* Figure recreated from Cambridge University Pathology Department. Available: http://www.path.cam.ac.uk/~schisto/general_parasitology/Digenean.lifecycle.gif

Trematodes display considerable variation in final hosts (e.g. fish, frogs, birds, crustaceans, mammals), but the molluscan intermediate stage is consistent among all species. It is therefore surprising that most focus tends to be on the final, typically vertebrate, host.

The prevalence of trematodes in molluscan hosts will vary from habitat to habitat but typically anywhere between 4% and 65% of molluscs are infected in any one locality and it is not uncommon for the majority of the weight of a field-collected mollusc to consist of trematode tissue (Esch *et al*., 2001; Lefcort *et al*., 2002; Morley, 2006).

Due to the nature and intensity of the relationship, trematodes have a major impact on a mollusc's physiology and immunology. Trematodes deploy a range of strategies to avoid detection and elimination by the immune system of the host at each stage of development (Morley, 2006). The severity of an infection is dependent on the parasite species and there are large differences in compatibility, even within species. Mortality rates in infected molluscs are often high due to tissue damage which

occurs upon infection or upon release of the cercariae, both of which are extremely traumatic events and far in excess of what any vertebrate host would experience. Death may also occur as a result of nutrients being diverted from the host to the parasite. Molluscs which survive infection are typically castrated by the parasite to increase the availability of nutrients (Morley, 2006).

There are more than 40 species of trematode which can utilise humans as a definitive host, these include: The Oriental lung fluke *Paragonimus westermani*, the liver flukes *Clonorchis sinensis*, *Fasciola hepatica* and *Opisthorchis viverrini* and the intestinal flukes *Fasciolopsis buski*, *Heterophyes heterophyes*, and *Metagonimus yokogawai* (Healy, 1970). These human-infective trematodes have in common the fact that they are acquired accidentally/indirectly from contaminated food, with one exception. Certain species of the genus *Schistosoma* are *directly* infective to humans *via* freshwater, and this is perhaps the reason that from a human health perspective, they are the most significant and best studied trematodes.

1.1.2. Species and distribution of Schistosomes

Transmission of human Schistosomes (Schistosomiasis) has been reported for 78 countries in total, of these it is endemic to 52, where transmission is moderate to high level (WHO, 2015).

Schistosomiasis predominantly occurs in tropical and sub-tropical areas, but the disease is also seen relatively frequently in travel clinics in Europe and North America among migrants and returning travellers who become infected after swimming in lakes (Knopp *et al.*, 2012).

There are two major types of Schistosomiasis in humans; intestinal and urogenital, caused by five main species (Table 1.1).

Table 1.1. Medically important human-infective species of the genus *Schistosoma. The adults of these species inhabit the veins of either the intestine or bladder of the human host. Other human-infective species exist but are so rarely contracted as to not warrant particular attention from a medical perspective.* Table adapted from: WHO.int, (2015). Available: http://www.who.int/mediacentre/factsheets/fs115/en/.

Form of disease	**Species**	**Geographical distribution**
Intestinal	*S.mansoni*	Africa, the Middle East, the Caribbean, Brazil, Venezuela and Suriname
	S.japonicum	China, Indonesia, the Philippines
	S.mekongi	Several districts of Cambodia and the Lao People's Democratic Republic
	S. intercalatum	Rainforest areas of Central Africa
Urogenital	*S.haematobium*	Africa and the Middle East

Schistosoma haematobium, *Schistosoma mansoni*, and *Schistosoma japonicum* are considered to be the three main species of interest/concern. *S.haematobium* and *S.mansoni* both occur in Africa and the Middle East, whereas only *S.mansoni* is also present in the Americas. *S.japonicum* is localised to Asia, primarily the Philippines and China. The remaining species are much more localised, with *S. intercalatum* occurring mainly in rainforest areas of Central Africa and *S.mekongi* more or less confined exclusively to the Mekong river basin (Colley *et al*., 2014; Figure 1.2).

85% of all infections occur on the African continent, where infection is so common that historically the haematuria (blood in urine) associated with *S.haematobium* was considered the male equivalent of menstruation (Rutherford, 2000).

Schistosoma mansoni is the most widespread species and infects more than 100 million people living in sub-Saharan Africa, the Caribbean, and South America (Negrão-Corrêa *et al*., 2012).

In Brazil, infection with *S.mansoni* remains one of the most important public health problems. The parasite infects as many as 6.8 million people across 9 states, with almost 43 million people at risk despite a higher degree of economic development when compared with other transmission countries, as well as more than 30 years of control programs (Barboza *et al*., 2012; Souza *et al*., 2012).

Although often considered primarily a rural problem, Schistosomiasis today is found even in urban regions; for example, a survey of children living in a neighbourhood of the Brazilian city of Salvador found that 30% were infected despite most having never left the city (Souza *et al*., 2012).

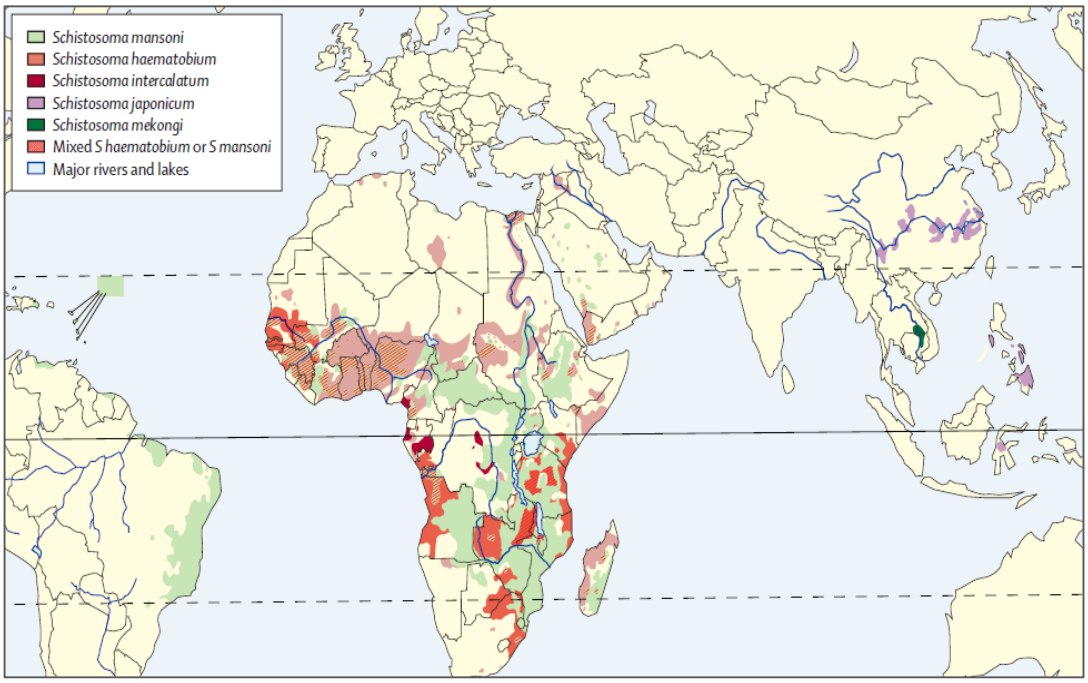


Figure 1.2. Global distribution of the five human-infective *Schistosoma* species of medical significance. *Shading represents areas where human infection is known to occur, with Schistosoma mansoni transmission foci in green; Schistosoma haematobium in pink; Schistosoma japonicum in lilac; S. intercalatum in dark red; S.mekongi in dark green and areas where both S.mansoni and S.haematobium occur together represented as dashed/orange.* Figure reproduced and adapted from Gryseels *et al*., (2006) and Colley *et al*., (2014).

Due to its significant distribution and medical importance I shall, for the remainder of this thesis, focus primarily on *S.mansoni*. Generally speaking, however, much of the information regarding the life-cycle is also applicable to the other species of human Schistosome.

1.1.3. Evolutionary history of *S.mansoni*

Unlike their snail hosts, Schistosomes have no calcareous or hard body parts, meaning that they have left no fossil record. Therefore, it is relatively difficult to accurately identify when the genus originated (Morgan, *et al*., 2001).

Early reports suggested Africa as the origin for the genus *Schistosoma*, but molecular phylogenetic studies suggest that the genus originated in Asia and migrated to Africa around 15 million years ago *via* mammalian migration, well after the fragmentation of the Gondwana supercontinent (Morgan *et al*., 2001; Beer *et al*., 2010). After reaching Africa, the descendants underwent considerable radiation and *S.mansoni* likely evolved in east Africa around 300-400 thousand years ago following the arrival of *Biomphalaria* from South America, as evidenced by the substantial genetic diversity of the parasite specimens in this region (Morgan *et al*., 2005).

S.mansoni is thought to have subsequently colonized South America as recently as 200-500 years ago with the transport of African slaves and was able to prosper due to the presence of an extremely susceptible host in *B.glabrata* (Morgan *et al*., 2001; Morgan *et al*., 2005). Only seven haplotypes have been found in South America and all are closely related and have West African affinities (Morgan *et al*., 2005).

S. mansoni is thought to be very effective in colonizing new areas and can do so without significant loss in genetic diversity, examples of this ability exist even in modern times. In the 1980's, following the construction of two dams and the associated appearance of vector snails, an *S.mansoni* outbreak occurred in an area of Northwest Senegal which had previously been free of reported transmissions (Picquet *et al*., 1996). Within a few years' infection rates in the local population were as high as 91% (Picquet *et al*., 1996).

A recent genetic study of the parasites from this region of Senegal showed a high level of diversity at both the nuclear and mitochondrial level, indicating the simultaneous emergence of parasites from several sources, something which the species is able to achieve and that enhances its ability to cope with selective pressures such as environmental conditions or even drug treatments (Van den Broeck *et al*., 2015).

1.1.4. *Schistosoma* life-cycle

Once eggs are released into freshwater in the faeces or urine of the infected human host, or in some cases that of certain domestic or wild animals which can act as reservoir hosts, a free-living miracidium hatches from each egg (Zahoor *et al*., 2010, Figure 1.3). Almost immediately after hatching, the parasites use the hair-like cilia surrounding the body to beat in unison and propel them forward at considerable speed in search of a suitable host snail and guided by a chemo-orientation response to glycoconjugates emitted from the snail's mucous surface layer (Haberl *et al*., 1995). The same glycoconjugates are also thought to stimulate the parasite's behaviour after contact with the snail (Kalbe *et al*., 2004).

After penetrating the snail, the parasite undergoes morphological and physiological changes, such as the shedding of the ciliated plates and transforms into a relatively immobile sack-like structure known as a primary or 'mother' sporocyst (Negrão-Corrêa *et al*., 2012). Accompanying these changes are the release of a variety of molecules from the parasite surface, these are referred to as excretory-secretory products or ESPs (Lodes and Yoshino 1990). ESPs generally consist of antioxidant enzymes, protease inhibitors, cysteine proteases and glycolytic enzymes (Lodes and Yoshino, 1990; Connors *et al*., 1991; Zelck and Von Janowsky, 2004; Guillou *et al*., 2007; Humphries and Yoshino, 2008 and Wu *et al*., 2009). These parasite-derived molecules can influence the behaviour of the host snails' defence cells to allow the parasites' survival. After 2- 3 weeks, through asexual reproduction, the mother sporocysts generate secondary or 'daughter' sporocysts which steadily migrate through the host tissue. Eventually, between 4 and 6 weeks after infection, the second free-living 'cercarial' stage is liberated from the daughter sporocysts and out of the snail into the aquatic environment and in search of the final host. Like the miracidial stage, cercariae are non-feeding and rely on glycogen stores for energy and consequently they have a limited period of infectivity of between 8-12 hours. Unlike miracidia, individual cercariae are sexually differentiated.

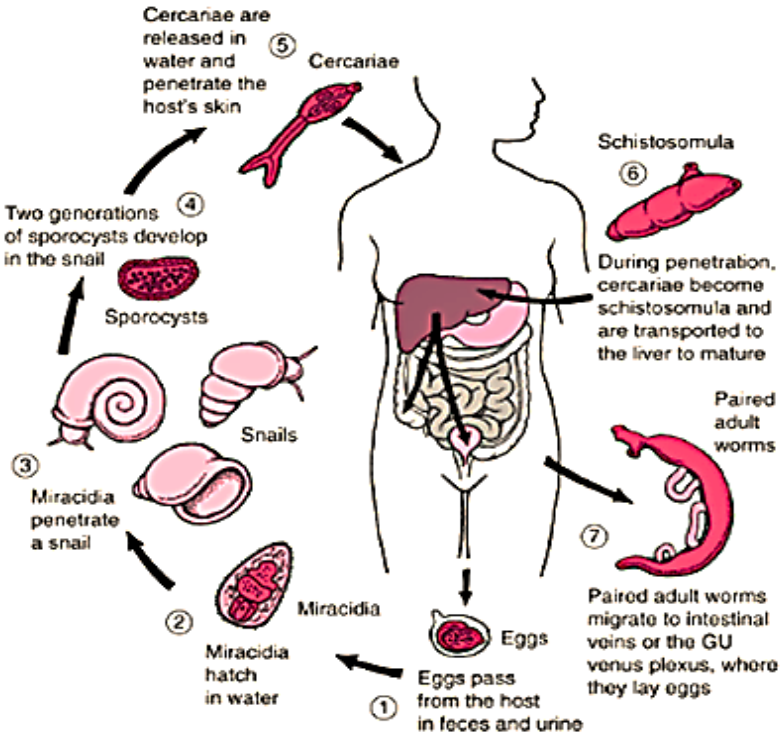


Figure 1.3. The life cycle of the three most prevalent human-infective *Schistosoma* species; *S.mansoni, S.haematobium* and *S.japonicum*. *Stage 1 occurs in both the human host and in the aquatic environment; stages 2-5 occur in the aquatic environment and/or a snail intermediate host; the three snails represent the different species which act as intermediate host for each of the three Schistosoma species; stages 6-7 occur exclusively inside the human host*. Figure reproduced and adapted from (Pearson, 2013). Available: http://www.merckmanuals.com/media/professional/figures/INF_schistosoma_life_cycle.gif

Recognition and attachment to the human host is stimulated by heat and the presence of the amino acid L-arganine. After attachment, the parasite loses its tail and releases enzymes from its penetration glands allowing it to pass through the skin (Haas *et al.*, 2002). Inside the host the parasite once again undergoes profound structural changes, including the development of a multi-laminate plasma membrane, and is now referred to as a Schistosomula. After a period of time the Schistosomula migrates through the blood stream to the lungs and subsequently to the liver to mature (Wakelin, 1996).

Adult male and female worms inhabit either the vasculature of the urinary plexus or the mesenteric venules surrounding the large intestine, depending on the species, where they mate and remain together *in copula* producing hundreds of eggs every

day and living between 3-10 years, but possibly as many as 40 (Taft *et al*., 2009; Colley *et al*., 2014). Eggs are either shed into the environment through faeces or urine allowing the cycle to continue. However, some eggs are not shed and instead remain within the host, this is the aspect of the life-cycle which produces the most serious pathology (from a human perspective). Retained eggs provoke an inflammatory reaction which can develop into a large granuloma, leading to hepatosplenamegoly and other serious, possibly fatal, conditions.

Because of their lack of immediate medical or veterinary significance, the life-cycle stages of digenetic trematodes occurring inside molluscs typically receive considerably less attention than do stages occurring in vertebrate hosts, when in reality alterations at any point in the cycle will ultimately be of equal importance (Wiley, 1992). Of all the people infected with Schistosomes each share the fact that they contacted freshwater harbouring infected snails, indeed, Schistosomes are obligatory parasites of freshwater snails, without which, no person could become infected and it is for this reason that the Schistosome-snail interaction is a bio-medically relevant target for research (Bayne, 2009).

To place subsequent chapters of this thesis into context and to serve as a reference, it is important to provide a more detailed explanation of the 'intra-molluscan' or 'snail' stages of development, on which much of the focus will be.

1.1.5. Biology of snail stages

Upon penetration of the snail intermediate host, the free-living miracidia undergo dramatic morphological and physiological changes resulting in the mass-replication and release of the human-infective cercariae stage (Wu *et al*., 2009).

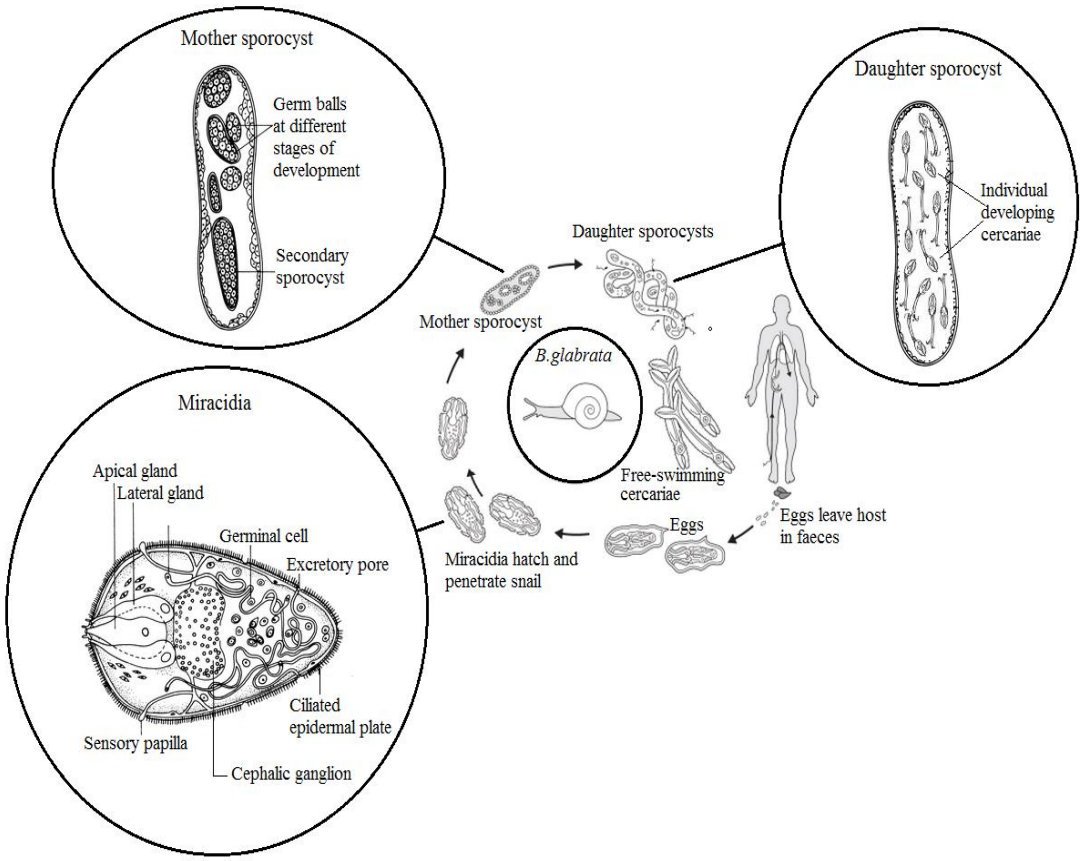


Figure 1.4. A closer look at the physiology of the Intra-molluscan stages of *S.mansoni* development. *The stages of the life cycle not infective to humans and which occur within the snail host, B.glabrata, are referred to as intra-molluscan; these include the miracidia, which begins this stage of the life cycle by penetrating the snail. After penetration the miracidia transforms into a mother sporocyst. Inside each mother sporocyst multiple daughter sporocysts develop via asexual reproduction.* Adapted from Farrar *et al*., (2014) and http://enconv.org/docs/index-23430.html

1.1.5.1. Miracidia; contact and penetration

The miracidium is the first free-living stage of the *S.mansoni* life-cycle and leaves the egg fully formed. Glycogen stores provide the parasite with a 12-hour energy source and since it has no means of feeding it must find an appropriate host before these reserves run out. While it may live for up to 48 hours, it will lose the ability to penetrate the snail much earlier (Toledo and Fried, 2014).

Upon locating a suitable host using chemical trails emitted by the snail, the miracidia will begin to probe the soft tissue, typically the tentacles, head-foot or mantle and will begin the process of attachment and penetration using specialised cellular structures (Toledo and Fried, 2014).

1.1.5.1.1. Epithelial cells

Most of the outer surface of the miracidium is covered with hair-like cilia, all of a similar length, arising from twenty-one flattened epidermal cells (also known as plates) which are arranged in four tiers (Figure 1.4). The cilia of the anterior tier are more rigid and numerous (Wajdi, 1966).

1.1.5.1.2. Papilla

The papilla are cone-shaped structures located at epithelial cell junctions. They are free of the cilia which cover the rest of the body and are considered part of the penetration apparatus, due to the presence of pores which discharge enzymes (Wajdi, 1966).

1.1.5.1.3. Adhesive glands

The apical and lateral adhesive glands, previously also called penetration glands, are single-cells which are slender with a bulb-like posterior and have a distinct pore, they are thought to be functionally analogous to the suckers of the adult worms (Wajdi, 1966; Kinoti, 1971). These glands secrete a mucus-like substance which allows the miracidium to remain attached to the snail tissue and possibly also secrete a lubricant to aid entry into the host during the penetration process (Wajdi, 1966).

1.1.5.1.4. Penetration gland

The penetration gland is a flask-shaped structure originally believed to be a primitive mouth/gut, but this theory has been discounted due to the lack of a lumen or any ability to ingest, rather it appears to secrete digestive enzymes to aid with host penetration (Wajdi, 1966).

1.1.5.2. Inside the snail

Penetration of *B.glabrata* by *S.mansoni* miracidia occurs with equal success regardless of the susceptibility status of the host, but once inside the differences in susceptibility may become apparent (Rizk *et al*., 2011).

As well as cilia, the surface of the miracidia is also covered in an array of glycotopes which differ starkly between individual parasites and between different stages of the same parasite (Peterson *et al*., 2009; Prasanphanich *et al*., 2013). These glycotopes are perhaps the primary promotor of the host defence response, although in certain circumstances some glycotopes, such as *S.mansoni* polymorphic mucins (*Sm*PoMucs), are thought to resemble those displayed on *B.glabrata* immune-cells and therefore benefit the parasite by serving as a form of molecular mimicry (Knight *et al*., 2014; Mickum *et al*., 2014; Adema and Loker, 2015). The heterogeneity of these glycotopes is believed to be at least partly responsible for the parasites compatibility with the host. Indeed, if the parasite is incompatible with the host it will be quickly detected and often faces swift encapsulation and death at the miracidial stage or soon after (Rizk *et al*., 2011).

1.1.5.2.1. Development to mother sporocyst

If the parasite proves to be compatible and avoids the snail immune response it will begin transformation into the mother sporocyst at, or near to, the penetration site, following shedding of the ciliated plates, which are readily phagocytosed by the snail immune cells (Farrar *et al*., 2014). After shedding plates, the expansion of the miracidial inter-epidermal ridges occurs and cells inside the parasite begin to secrete cytoplasmic and lipid membranes which spread over the surface to form a ‘syncytial tegument’ (Peterson *et al*., 2009; Bayne, 2010). The whole process of transformation occurs over about 48-hours post-penetration, but sporocysts may be identifiable after only a few hours (Meuleman *et al*., 1978). Amongst other factors, heat shock protein 70 (Hsp 70) is thought to play an important role in the transformation of the miracidia into sporocysts, as displayed by the ability to induce miracidial transformation *via* bombardment of Hsp70-coated particles (Heyers *et al*., 2003).

The stage between shedding the plates and final transformation to mother sporocyst is perhaps the most vulnerable time for the parasite and is often when encapsulation and death occurs, not least since the mimicry glycotopes are initially lost with the shedding of the plates (Peterson *et al.*, 2009). Despite the initial loss of the plates and potentially also the *Sm*PoMuc 'smoke-screen', the parasite may still be granted a degree of protection due to the release of excretory-secretory products (ESPs) which, like *Sm*PoMucs, serve as a trigger for the immune response in resistant snails, but have also been shown to interfere with a range of internal defensive capabilities in susceptible animals (Wu *et al.*, 2009; Coustau et al., 2016).

1.1.5.2.2. The mother sporocyst

Even in compatible hosts, only a small number of miracidia typically develop into mother sporocysts (Cook *et al.*, 2009). At around 96-hours post-penetration the mother sporocyst is an elongated sac-like structure filled with germinal cells and small vacuoles, which have budded from the epithelial lining, and is ~200µm in length, about the same size as the miracidia (Wajdi, 1966; Cook *et al.*, 2009). Like the miracidia, it has no digestive system, but unlike the miracidia it is able to absorb nutrients directly across its thin syncytial cytoplasmic outer-surface (Wakelin, 1996). By the end of the first week the parasite has grown considerably and the proliferation of germinal tissue is extensive enough to fill the whole body cavity and in some cases the sporocysts can be seen by eye as tumour-like bulges in the snail tissue (Wajdi, 1966).

As well as allowing nutrient absorption and osmoregulation, the tegumental surface of the sporocyst also forms the parasites primary defence against the host immune response (Johnston and Yoshino, 1996). Much like the miracidia, the sporocyst makes use of various glycotopes designed to make the parasite essentially invisible.

The germinal cells inside the sporocyst continue to grow and divide to form second-generation or 'daughter' sporocysts. The daughter sporocyst embryos are enveloped by a basic epithelial layer derived from the mother, under which somatic cells expand, which eventually degenerates to expose a new tagumental surface layer (Meuleman *et al.*, 1980).

1.1.5.2.3. Development of daughter sporocysts

After around 8-15 days' post-penetration, the mother sporocyst uses muscular contractions, facilitated by exogenous serotonin, to allow the emergence of hundreds of daughter sporocysts, which begin to migrate toward the snails' digestive gland and gonads (Delgado *et al.*, 2012; Farrar *et al.*, 2014). Inside the daughter sporocysts further germinal cells develop, much as they did inside the mother sporocyst.

At this stage of development, the daughter sporocysts begin to release a factor known as schistosomin from their teloglial cells (De Jong-Brink *et al*., 2001b). Schistosomin is thought to be responsible for the interruption of the hosts endocrine system by halting reproduction and growth and diverting resources toward parasite development (De Jong-Brink *et al*., 2001b).

1.1.5.2.4. Cercariae; development and release

After around 2 weeks residing in the digestive gland-gonads, the final intra-molluscan stage has formed. The 'cercariae' develop in a similar manner to the daughter sporocysts, with somatic cells developing inside a daughter sporocyst-derived epithelial layer (Meuleman and Holzmann, 1975). The asexual reproductive process from mother to daughter, and to the final stage, means many thousands of cercariae can be produced from a single miracidium (Cook *et al.*, 2009). Once fully developed, the cercariae emerge in minutes from the daughter sporocyst, head first, and propelled by lashing of the tail, after which the sporocyst wall quickly heals (Wajdi, 1966; Hansen, 1975). At this stage, daughter sporocysts are also capable of halting cercarial production to instead produce additional generations of daughter sporocysts (Jourdane *et al*., 1980).

Once outside the daughter sporocyst, free cercariae again make use of molecular masking to avoid destruction and are carried by the snails' circulatory system to several vascularized peripheral locations, particularly the edge of the mantle and the pseudobranch (Hansen, 1975; De Jong-Brink *et al*., 2001b).

Upon reaching a suitably exposed location, the cercariae wait for temperature and light cues which signal mid-day, at which point they burst out of the snail and into

the aquatic environment (Toledo and Fried, 2014). Many thousands of cercariae can emerge from the snail at one time to go in search of the final host.

Having looked at *S.mansoni,* and focusing on the often over-looked snail stage of its life-cycle, it is equally important that we understand the biology of the snail host as this is one of the most decisive stages in determining the death or survival of the parasite and the extent of subsequent transmission to humans (Soomro, 2005).

1.2. *Biomphalaria*

Most intermediate hosts of human *Schistosoma* parasites belong to three genera of gastropods: *Biomphalaria, Bulinus* and *Oncomelania*.

Pulmonate snails of the genus *Biomphalaria* are the most common freshwater intermediate Schistosome host snail prevailing in developing countries, a fact that is responsible for the wide distribution and subsequent medical importance of the parasite (Bakry, 2009).

1.2.1. *Biomphalaria glabrata*

Biomphalaria glabrata snails are important intermediate hosts in the transmission of *S.mansoni* in the New World and more recently also in Egypt due to exotic introduction into canals, possibly as early as 1981, and there is evidence that it is hybridizing with the native *B.alexandrina* (Kristensen *et al*., 1999; Humphries and Yoshino, 2006). As *B.glabrata* has proven such an excellent host for *S.mansoni,* it is likely that its presence would only favour increased transmission in these areas (Morgan *et al.*, 2001).

When infected African slaves were first brought to Brazil they were placed around coastal areas which were also inhabited by very susceptible host snails (*B. glabrata*) (Morgan *et al.*, 2001). Since the time of these original introductions, massive anthropogenic changes, such as damming, to the same environments, have enabled the spread of *B. glabrata* and other indigenous *Biomphalaria* species (Morgan *et al.*, 2001).

Biomphalaria glabrata is of interest in terms of transmission dynamics, but increasingly it is used as a model for the study of the complex host-parasite interaction and innate immune response (Baeza *et al*., 2010).

Considering the growing body of literature on its biology, especially its immune system and response to infection, and the progress of the assembly of the genome which has been sequenced and is, as of late 2015, undergoing annotation (Coustau *et al*., 2015), coupled with the lack of existing models for molluscs, *B. glabrata* is now emerging as a candidate model species (Guillou *et al*., 2007). As well as improving its status as a model organism, our ever-increasing understanding of the genetic processes underpinning the parasite-host relationship will likely increase and improve the tools available to researchers looking toward control solutions and even cures (Knight *et al*., 2014; Giannelli *et al*., 2015).

1.2.1.1. Evolutionary history and distribution

Biomphalaria glabrata was first described by the American naturalist Thomas Say, under the name *Planorbis glabratus*, based on a shell given to the Academy of Natural Sciences of Philadelphia in 1818 which was allegedly discovered in the US state of South Carolina (Paraense, 2001). Since no subsequent snails could be found in South Carolina it is thought that the shell was actually from Guadeloupe, since the person who donated the original sample also spent considerable time there and it is a location we now know to be part of the species natural distribution (Paraense, 2001).

Regarding its evolutionary history, *B. glabrata* was originally believed to have first appeared 100 million years ago on the super-continent of Gondwana and that the present species in Africa and South America are a result of the continental split which occurred 70 million years ago (Davis, 1980).

Using modern genetics tools, DeJong *et al*., (2001a) definitively demonstrated that the South American species are basal and the African species were derived from them (Figure 1.5). This suggests that the genus originated in South America after the continents had separated and that a proto-*Biomphalaria glabrata* subsequently made the transatlantic crossing, possibly in the feathers or stomach of a bird. The case is further strengthened by the finding that the African species of *Biomphalaria* have

only a quarter of the mitochondrial genetic diversity of *B. glabrata* (DeJong *et al*., 2001a). Based on African fossil records, this is thought to have occurred between 2-5 million years ago (Morgan *et al*., 2001).

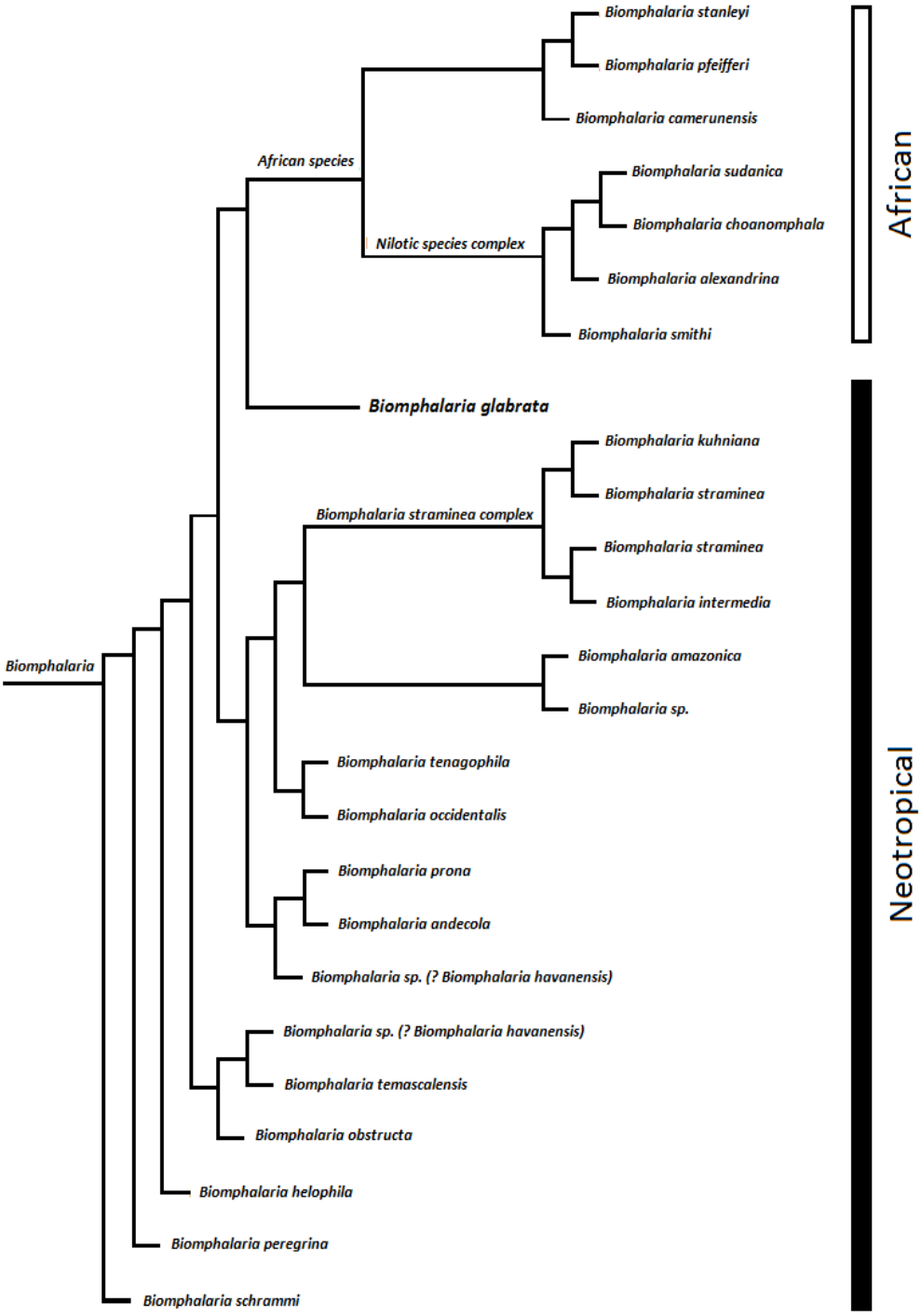


Figure 1.5. Phylogenetic relationships within and between the African and American species of the genus *Biomphalaria. This tree is based on combined mitochondrial and nuclear sequence data collected by Dejong et al., (2001a).* Figure recreated from Dejong *et al*., (2001a).

Within *B.glabrata,* five differentiated clades can be identified and are referred to by their geographic origin, these are: the Greater Antilles, the Lesser Antilles, Venezuela and two clades in Brazil which are geographically overlapping (Dejong *et al*., 2003; Figure 1.6). The different Brazilian clades likely arose as a result of habitat fragmentation but, based on the overlapping distributions, parapatric speciation is also possible (Dejong *et al*., 2003). The divergence of lineages within these American clades probably occurred around 740,000 years ago.

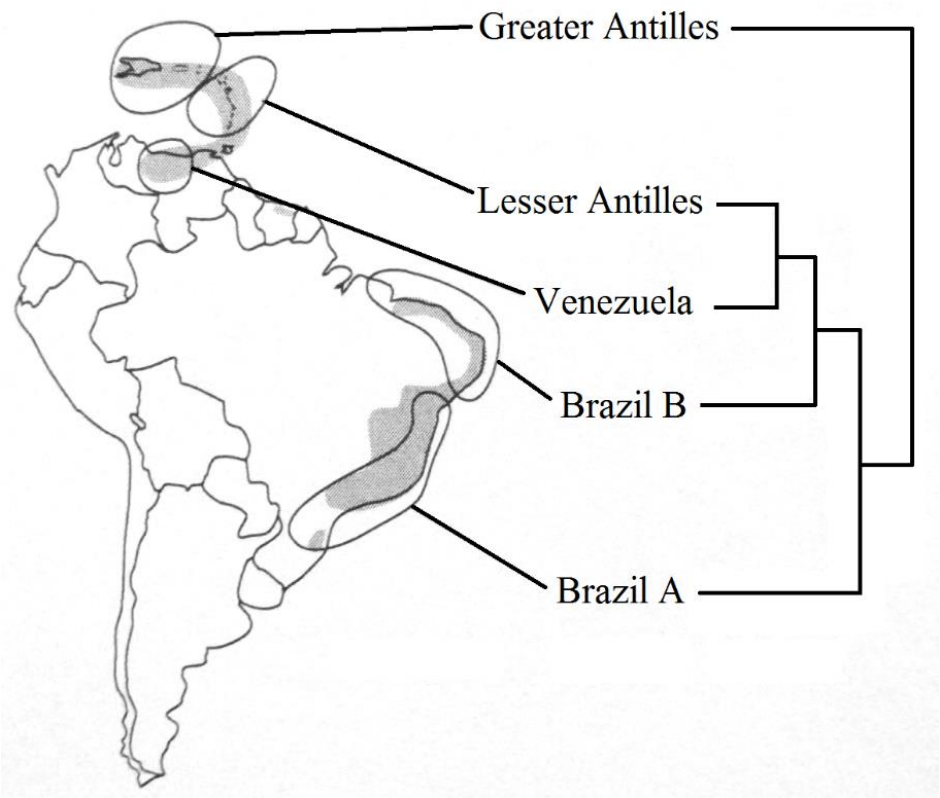


Figure 1.6. Geographic distribution of the phylogenetic relationships among the five clades of the species *Biomphalaria glabrata*. *The current distribution of B.glabrata is represented by shading and diagonal lines indicate the areas from which snails were collected for use in mitochondrial and nuclear sequencing.* Figure reproduced from Toledo and Fried, (2010), data based on the work of Dejong *et al*., (2003).

In South America the native distribution of *B.glabrata* extends from southern Brazil, through French Guiana, Suriname and Venezuela (Pointer *et al*., 2005). The snail also extends throughout the Lesser Antillies in Martinique, Guadeloupe, Dominica, Antigua, Saint Martin, Saint Kitts and the Greater Antillies in Puerto Rico, Haiti and the Dominican Republic (Pointier *et al*., 2005).

B.glabrata is also now resident in Egypt having been introduced around 1981 (Pointer *et al*., 2005). Snails must be in, or close to, a source of still or slow-moving freshwater. As a result of these requirements they are often found in ponds, swamps, ditches, streams, reservoirs and even sewage dump sites (Jurberg *et al*., 1997; Freitas, 1976).

1.2.1.2. BBO2

While natural populations of *B.glabrata* are known to be polymorphic in their degree of resistance to *S.mansoni*, laboratory studies typically rely on specific snail strains which have been inbred and maintained for certain traits (Ataev and Coustau, 1999; Giannelli *et al*., 2015).

One of the most widely used experimental strains of *B.glabrata* is known as BBO2.

BBO2 snails are a susceptible field isolate which were originally collected in 2002 by Omar dos Santos Carvalho from the district of Barreiro in the Brazilian city of Belo Horizonte. BBO2 signifies '*Biomphalaria*, Barreiro, 2002' (Adema *et al*., 2006).

These snails were found inhabiting a small stream in a metropolitan area, on a ranch where flowers were grown (http://biology.unm.edu/Biomphalaria-Genome/BB02STRAIN.html).

BB02 has since been distributed throughout the world by various research departments and is being seen as a model organism among molluscs as *Drosophila melanogaster* is for insects and *Caenorhabditis elegans* for nematodes (Knight *et al*., 2002).

1.2.1.3. Biology

As the genus *Biomphalaria* has many traits which make it distinct among molluscs, so *Biomphalaria glabrata* has traits which differ from others in the genus. Some of these differences are of considerable importance as they relate to its success as a vector of *S.mansoni*, both in terms of compatibility but also in its ability to colonize new areas and survive. The effectiveness of the snail as a vector provides its primary

interest among researchers and much of this interest is focused on molecular level compatibility. While molecular parasite-host compatibility is fundamentally important, it is also necessary to understand the general biology of the species to understand where and how these differences actually occur.

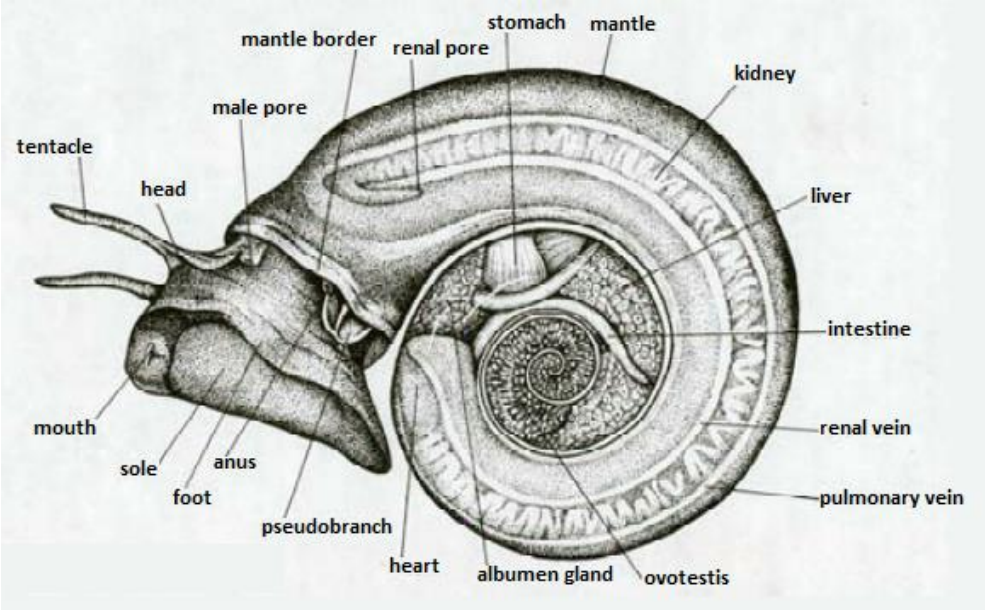


Figure 1.7. Anatomical diagram showing the basic body plan of *B.glabrata. Major organs and functional structures are labelled and their approximate location indicated by straight lines*. Figure reproduced and adapted from Malek, (1985).

1.2.1.3.1. Reproduction

B. glabrata is a simultaneous hermaphrodite, capable of both self and cross-fertilization, the latter method typically being preferred (Eveland and Haseeb, 2011). The reproductive tract is subdivided into three parts: hermaphroditic, male and female (Jarne *et al*., 2011). There is little preference between mating roles and an individual snail will change from male to female (or *vice versa*) in about 45% of its cross-fertilization events (Vernon and Taylor, 1996).

Oocytes are produced in the ovotestis and are subsequently transported *via* the spermoviduct to the carrefour (pouch), where fertilization occurs (Eveland and Haseeb, 2011). Glactogenic perivitelline fluid is synthesised and secreted by the albumen gland to surround each egg cell as protection and nutrition for the developing embryo (Bai *et al.*, 1996).

A mature snail can lay as many as 10,000 eggs in a year and these eggs are laid in masses surrounded by a thick double-walled gelatinous membrane. The masses are gelatinous to protect against short periods of desiccation and consist of around 30-40 eggs firmly adhered to any item which is smooth and suitably submerged, including the shells of other snails (Eveland and Haseeb, 2011). Mature snails are capable of laying one mass per day (Pimentel, 1957).

Embryos go through a series of complex developmental stages beginning with the blastulae 0–15 hours after the first egg cleavage, followed by the gastrulae (24–39 h), the trocophore (48–87 h) and finally the veliger beginning at around 96–111 hours (Camey and Verdonk, 1969).

Snails will hatch after 6-8 days and will reach reproductive maturity by 4-7 weeks (WHO, 1995).

1.2.1.3.2. Respiration

Respiration in *B.glabrata* is usually attributed to actions within the mantle cavity, the tegument and the pseudogill (Paraense, 1972).

If the snail is unable to surface it can switch from aerial to aquatic respiration and remain submerged for up to 92 days, in the opposite situation it is able to survive for up to 16-hours, without the dissolved oxygen provided by water (Jurberg 1992; Jurberg *et al.,* 1997).

Distinguishing the exact contribution of a particular structure to the respiratory process has proven difficult for numerous reasons. There is no histological difference between aquatic and aerial-related respiratory epithelia; *B.glabrata* fills its mantle cavity with air *via* a pneumostoma, something which is characteristic of aerial respiration, yet it also performs gas exchanges when submerged, which is indicative of aquatic respiration (Jurberg *et al.,* 1997). Furthermore, the air-bubble inside the mantle, which serves as a flotation aide and oxygen store, along with circulating water, is thought to act like a physical gill (Jurberg *et al.,* 1997). Essentially, *B.glabrata* is able to perform a kind of aquatic respiration, but while it has the morphophysiological properties needed to perform the functions of a typical physical gill this has proven difficult to demonstrate experimentally.

The mantle of *B.glabrata* contains three chambers related to respiration: the air chamber and the water inflow and outflow chambers (Jurberg *et al.,* 1997; Figure 1.8). The intra-mantle water flow is also believed to help with the removal of waste and acts as an internal structural support for the shell, allowing the snail to descend to greater depths without the shell succumbing to pressure fractures (Jurberg *et al.,* 1997).

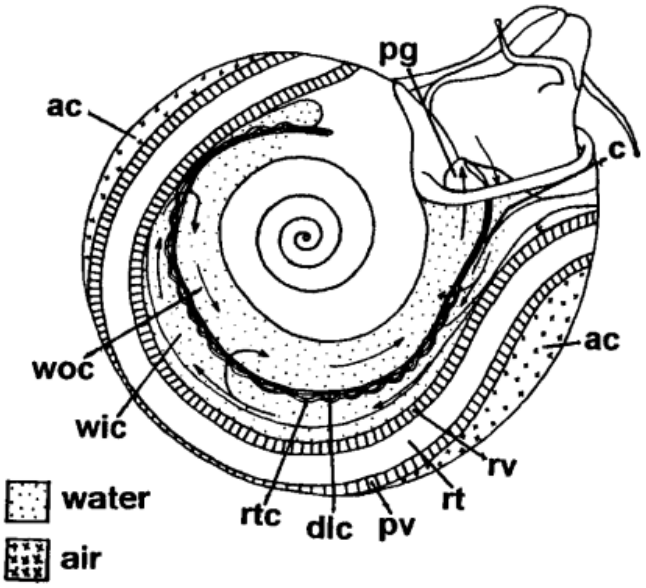


Figure 1.8. Anatomical diagram of the respiratory process of *B.glabrata* and the proposed role of water-flow. *WOC = Water outflow chamber, WIC = Water inflow chamber, ac = air chamber, c = crack, pg = pseudogill, rtc = rectal cristae, dlc = dorsolateral cristae, rv = renal vein, pv = pulmonary vein, rt = renal tube. Arrows indicate direction of water flow.* Reproduced and adapted from Jurberg, Cunha and Rodrigues, (1997).

The respiratory pigment in most invertebrates, including the vast majority of gastropods, is hemocyanin. This protein is synthesized and secreted by rhogocytes (pore cells) and gives the hemolymph a distinctive blue colour (Kokkinopoulou *et al*., 2014). Interestingly, there is one family of gastropods, the *Planorbidae*, which includes *B.glabrata*, in which the hemolymph is red in colour. Unusually for invertebrates the rhogocytes of the *Planorbidae* instead produce hemoglobin. The pigment performs much the same oxygen-carrying task as in vertebrates but is not bound to a cell (Lieb *et al*., 2006).

The divergence in respiratory pigment in this family is thought to be an adaptation to the hypoxic pond habitats of many species, since hemoglobin has a greater oxygen affinity than hemocyanin (Moeller *et al*., 2011). Since other gastropods, such as the

Lymnaeidae, occupy the same habitats, it is thought that hemoglobin is also advantageous to *B.glabrata* in allowing an increased diving potential (Lieb *et al*., 2006; Figure 1.9).

The hemolymph has been found to contain trace quantities of molecules morphologically similar to hemocynin, which perhaps demonstrates the evolutionary divergence (Lieb *et al*., 2006).

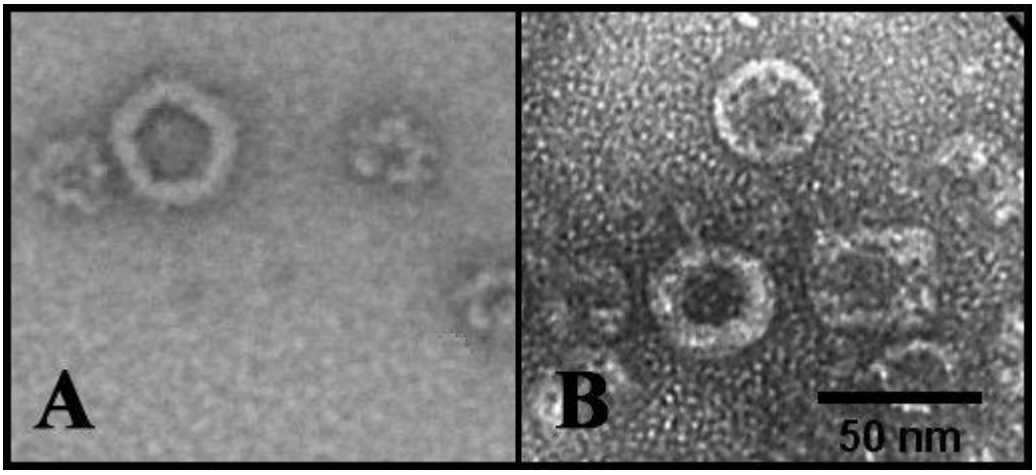


Figure 1.9. A comparison between the respiratory pigments of snails from the genus *Biomphalaria* and those from another, more typical, gastropod. *A) Haemoglobin from B.glabrata, B) Hemocyanin from the Keyhole limpet (Megathura crenulata).* Picture A reproduced and adapted from Lieb *et al*., (2006) and picture B reproduced and adapted from Becker *et al*., (2014).

1.2.2. *Biomphalaria* immune system

To fully understand the ways in which *S.mansoni* transmission can be determined by *B.glabrata* we must first understand what is perhaps the most important aspect of the relationship; the immune system of the vector. An overview of general invertebrate immunology and specifically of molluscs, is fundamental when putting into context the capabilities which *B.glabrata* has to influence transmission, especially since many of these are different to those which more familiar mammalian hosts possess. A mechanistic knowledge of what is a significant potential barrier to the parasites development toward human infection helps us to appreciate the possible influence of internal and external factors.

1.2.2.1. Overview of the invertebrate/molluscan immune system

For multicellular organisms at least, it is possible to distinguish two categories of immune response: the innate (or 'non-specific') and the adaptive (or 'specific). Recent research has suggested that this distinction is not as clear as once thought, for example: *B.glabrata* is capable of shifting and enhancing its anti-schistosome immune response, from cellular to primarily humoral, after repeated parasite exposure (Schmid-Hempel, 2007; Pinaud *et al*., 2016).

Invertebrates only possess the innate response, whereas vertebrates possess both. The innate system is often also referred to as 'basic' immunity and while there are valid reasons for this term, it does not mean that this type of immunity is *inferior*, or has been superseded, since invertebrates still make up 95% of all the animal species on earth (Rinkevich, 1999; Canesi *et al*., 2002; Cooper, 2006).

The study of innate immunity continues to be of extreme importance, not only because it is a component of the human immune response, but also because invertebrate species of medical importance rely on innate immunity to ensure their own survival. Furthermore, the economic importance of invertebrates both as pests and also as food continues to drive research in innate immunity processes (Canesi *et al*., 2002).

The fundamental principle of the invertebrate immune system is based on the ability to distinguish between 'self' and 'non-self' (foreign) objects. Recognition of a foreign object clearly provides the basis of an immune system and the ability to recognize its own tissue prevents an organism from attacking itself. Consequences of the failure of the latter process are revealed as autoimmune diseases, which are well known in humans (e.g. Chron's disease), but also occur in invertebrates (Schmid-Hempel, 2007).

Aside from the most primitive species, in which organs and functions are difficult to differentiate (e.g. sponges), invertebrates have an open circulatory system in which a fluid termed 'hemolymph' is contained in the body cavity or 'hemocel' and directly bathes the organs (Galloway and Depledge, 2001; Ottaviani, 2011). The hemolymph, which is similar in function to vertebrate plasma, generally contains immune components which include antimicrobial peptides, agglutinins and lysosomal enzymes. These components serve to enhance opsonization by facilitating bacterial

aggregation and immobilization and/or display cytolytic activities (Labreuche *et al*., 2006). Free-floating in the hemolymph are the principle effectors of the invertebrate immune response, referred to collectively as 'hemocytes' (Labreuche *et al*., 2006). These cells are in many respects analogous to vertebrate macrophages and are essential in the killing and clearance of any objects recognized as foreign, by a number of means (Fryer and Adema, 1993). As with macrophages, a vital function of hemocytes is the process of phagocytosis of any foreign particles which are small enough (De Vico and Carella, 2012). The phagocytic process is a sequence of distinct stages: attraction (chemotaxis), attachment, engulfment and digestion; a process that has been highly conserved throughout evolution due to its effectiveness (Bayne and Fryer, 1994; Fournier *et al*., 2000).

In more complex invertebrates such as the arthropods, annelids and molluscs, phagocytosis of larger objects, or large numbers of smaller ones, is supplemented by 'encapsulation', a process which could be considered analogous to granuloma formation in vertebrates (Galloway and Depledge, 2001).

The process of encapsulation has been described as "frustrated phagocytosis" (Bayne and Fryer, 1994). This is an apt description because the process of encapsulation is essentially the same as phagocytosis, indeed hemocytes will still attempt to phagocytose large objects but instead of succeeding they end up forming a multi-layered sheath of cells around the intruder (Pech *et al*., 1995).

The directed migration of cells toward specific stimuli (i.e. chemotaxis) is a central component of the immunological/inflammatory reaction throughout the animal kingdom. Invertebrates also respond to infection or injury by enhancing the motility of immunocompetent cells. Hemocytes from many invertebrate species have been shown to migrate non-randomly toward bacteria and other exogenous material (Raftos *et al*., 1998).

The molluscs represent one of the most diverse and species-rich phyla of the animal kingdom. With more than 130,000 known species they are second only to the arthropods in number (Gruner, 1993; Gruner *et al*., 1993). Of the seven extant molluscan classes, the gastropods make up more than 80% of total species, with bivalves constituting the major part of the remaining species at around 15% (Oehlmann and Schulte-Oehlmann, 2002).

Despite our familiarity with terrestrial molluscs they are primarily aquatic animals, six of the seven classes within the phylum are entirely aquatic and the majority of the species in the remaining class, the gastropods, are also aquatic (Oehlmann and Schulte-Oehlmann, 2002). Of the aquatic molluscs, most are marine but again, gastropods, along with bivalves, appear to show the most diversity with several species inhabiting freshwater environments (Oehlmann and Schulte-Oehlmann, 2002).

Molluscs have a coelomatic cavity, which makes it possible to distinguish well-defined cellular and humoral components within the immune system. Molluscan hemocytes may be found freely circulating in the hemolymph or may be 'fixed' in internal tissue such as the digestive gland (Ottaviani, 2006; Loker, 2010).

Molluscs, like other invertebrates, are able to discriminate between self and non-self, this has been demonstrated by transplant studies on freshwater species which will accept an autograft (of native/self-tissue) while rejecting, and eliciting an immune response against, a xenograft (foreign tissue) (Ottaviani, 2004).

Molluscan hemocytes are composed of different subpopulations, however the precise classification of these remains controversial (Blaise *et al*., 2002; Ottaviani, 2011). It can at least be said that the majority of mollusc species appear to have two types of principle hemocyte and these are distinguishable based on either their granularity or their ability to spread (Humphries and Yoshino, 2003; Ottaviani, 2006; Yoshino and Coustau, 2011). 'Spreading' refers to the behaviour of certain hemocytes *in vitro*. When placed on an artificial surface the cells typically spread out, forming conspicuous projections known as filopodia (Noda and Loker, 1989). Spreading cells are thought to represent the majority of hemocytes and are also thought to be the main cells involved in phagocytosis and encapsulation, since both processes require attachment and, in the case of encapsulation, spreading over the surface of the foreign object (Noda and Loker, 1989). In addition to phagocytosis and encapsulation, the molluscan hemocytes also release cytotoxic reactive oxygen and nitrogen intermediates which are directly responsible for the death of the captured organism (Walker, 2006).

Hemocytes in molluscs are also involved in wound healing, nerve repair, shell formation and repair (certain species), tissue remodelling and the movement of metabolites and nutrients (Noda and Loker, 1989).

1.2.2.2. Hemocytes of *B.glabrata*

Much of the information surrounding the circulatory/immunological system of *B.glabrata* remains controversial since studies have often failed to form a broad consensus and investigatory methods typically differ considerably.

The site of hemocyte origin is often reported as being the 'amebocyte producing organ' (APO), which is believed to act in a similar manner to the bone marrow of vertebrates (Barbosa *et al*., 2006). The presence of high levels of cellular mitosis and hyperplasia at this site, as well as its apparent ability to induce a degree of resistance when transplanted into susceptible snails, is said to offer support to this theory (Souza and Andrade, 2006). However, through a series of histologic, morphometric and ultrastructural studies, Barbosa *et al*., (2006) were unable to find significant evidence to support the APO as the central organ for hemocyte production.

Others suggest a 'multicentric' origin, with hemocytes being produced at various sites throughout the body in a manner similar to the formation of blood cells from endothelial lines in the early evolutionary stages of other animals (Souza and Andrade, 2006). This theory suggests that an APO is unnecessary, since hemocytes can be formed at different sites throughout the body (Souza and Andrade, 2006).

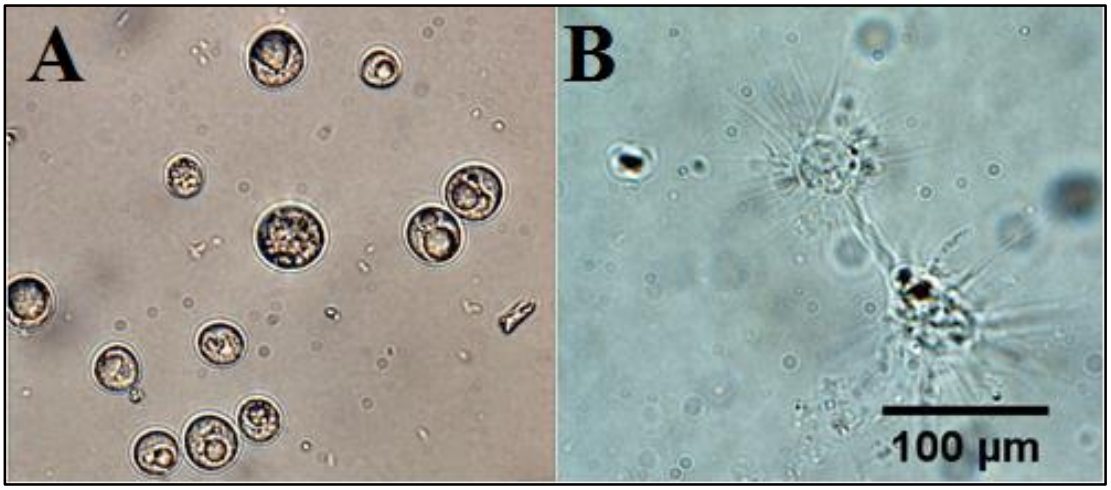
Unlike some other molluscs, the hemocyte population of *B.glabrata* appear to represent morphological and biochemical heterogeneity, which makes their classification extremely difficult and this is compounded by the fact that they also lack structures such as secretory granules which are normally used to aide classification (Delgado *et al*., 2001; Barçante *et al*., 2012). The hemocytes of *B.glabrata* therefore either represent a single type of cell at different stages of maturity, or several different sub-populations (Delgado *et al*., 2001).

While many hemocytes freely circulate in the hemolymph others, termed 'fixed' hemocytes, are spread throughout the connective tissue and are known to internalize and store foreign material, as well as migrating to infected and/or damaged sites

(Matricon-Gondran and Letocart, 1999). Historically, it has been assumed that free-circulating and fixed hemocytes only differ based on their location (and that they could presumably switch roles). However, it has also been suggested that free and fixed hemocytes are in-fact functionally independent and form two separate components (Borges *et al.*, 2006). If this is the case then, due to the way hemolymph is collected, the majority of hemocytes that have been studied thus far would be of the circulating kind. Nevertheless, circulating cells have themselves been shown to contain several sub-populations, depending on the study.

The majority of studies have settled on the statement that, as with other mollusc species, *Biomphalaria glabrata* hemocytes are composed of *at least* two distinct populations (Table 1.2). These are identified by either their propensity to spread, or otherwise remain rounded, on contact with an artificial surface (e.g. glass) *in vitro* or by their degree of cellular granularity. When classifying based on granularity, the two populations have been termed 'hyalinocytes' (smaller cells with a non-granular appearance) and 'granulocytes' (larger, granular cells). When classified based on spreading ability they are often simply referred to as 'spread' or 'round' (Figure 1.10). Alternative classifications have been reported and in these cases the cells are usually named according to their size.

Figure 1.10. Comparison between the two main functional states in which immunocompetent hemocytes of *Biomphalaria glabrata* are typically observed *in vitro. A) Rounded hemocytes B) Spread hemocytes on glass*. Pictures taken at 400x.



The next most common classification is of 3 hemocyte types, usually termed small, medium and large (Matricon-Gondran and Letocart, 1999; Martins-Souza *et al.*, 2009; Table 1.2). Delgado *et al*., (2001) reported 4 hemocyte types, these included hyalinocytes, 2 types of granulocyte and a newly reported 'erythrocyte' type which was thought to resemble the biconcave shape of the mammalian RBC. The largest number of cell types was reported by Cavalcanti *et al*., (2012) who describe 5 cell types; granulocytes, 3 types of hyalinocyte and blast-like cells which had a very large, centrally-located, nucleus (Table 1.2).

Table 1.2. An example of some of the different populations and compositions of *B.glabrata* hemocytes reported in the scientific literature. *Various studies report different numbers of uniquely distinguishable hemocyte populations within B. glabrata.*

Number of cell types	Name	Most numerous cell type	Reference
3	small, medium and large	Medium	Martins-Souza *et al*., 2009.
2	Granulocyte, hyalinocytes	Granulocyte	Harris, 1975.
2	Granulocyte, hyalinocytes	Hyalinocytes	Bakry *et al*., 2012b.
2	small and large haemocytes	Large	Barçante *et al*., 2012.
4	Granulocyte (types 1 and 2), hyalinocytes (type 3), erythrocyte type	Type 1 and 2 granulocytes (differs by snail strain)	Delgado *et al*., 2001.
2	Granulocyte, hyalinocytes	Granulocyte	Barracco *et al*., 1993.
3	small, medium and large	Medium and large almost equal	Matricon-Gondran and Letocart, 1999.
5	Blast-like, Granulocyte, hyalinocytes (types 1, 2 and 3)	Type I hyalinocytes	Cavalcanti *et al*., 2012.

Similar disagreement exists regarding the proportions of circulating cells that the different populations make up (Table 1.2).

Granulocytes were found to be the most common type of cell by Harris (1975) and Barraco *et al*. (1993). Delgado *et al*., (2001) also reported granulocytes as the main cell type but in their classification there were two types and the ratio differed according to snail strain. Both Cavalcanti *et al*., (2012) and Bakry *et al*., (2012) identified hyalinocytes as the most abundant cell type. Medium and large hemocytes have also been reported as the most populous circulating cell type (Matricon-Gondran and Letocart, 1999; Martins-Souza *et al*., 2009; Barçante *et al*., 2012). Due to the disagreement and difficulty in hemocyte classification many researchers prefer to pool the cells and treat them as a single type or to separate them based on their ability to spread.

1.2.2.3. Interaction with *S.mansoni*

Self/non-self discrimination in *B.glabrata* is achieved through pattern recognition receptors (PRRs) on the surface of hemocytes which recognise foreign structures referred to as pathogen-associated molecular patterns (PAMPs) (Janeway 1989; Janeway and Medzhitov, 2002; Yoshino and Coustau, 2011). Some of the most important and well-studied PRRs are fibrogen-related proteins (FREPs) (Coustau *et al*., 2016). FREPs are complex pattern recognition proteins and are particularly important due to their interaction with certain PAMPs, particularly the highly polymorphic mucins (*Sm*PoMucs) (Prasanphanich *et al*., 2013; Coustau *et al*., 2016).

Upon penetration of the host, proteins are secreted from the lateral and apical penetration glands of the miricidia and an array of larval proteins known as excretory-secretory proteins (ESPs) are released during the subsequent transformation to the primary sporocyst stage (Yoshino and Coustau, 2011). It is during this period that the proteins released serve as chemical signals to the host PRRs ‘announcing’ the parasite's presence, to which the host response is initiated (Yoshino and Coustau, 2011; Barçante *et al*., 2012). As mentioned in sections 1.1.5.2 and 1.1.5.2.1, while these ESP proteins serve as PAMPs, depending on the genetic compatibility between parasite and host, they may also act in favour of the

parasite by interfering (diverting, overwhelming or neutralizing) the immune response induced by the PRRs.

The ciliated plates shed by the transforming miracidia are rapidly phagocytosed by hemocytes (Bayne, 2009). The parasite itself, being too large to be phagocytosed, is instead encapsulated. This is the point at which we notice a difference between strains of *B.glabrata*. While some *B.glabrata* strains are susceptible to *S.mansoni*, allowing parasite development and reproduction, others are resistant and able to suppress the infection (Basch, 1976; Loker and Bayne, 1986; Humphries and Yoshino, 2006). Hemocytes from either resistant or susceptible snails will encapsulate sporocysts however, sporocysts within capsules formed by susceptible hemocytes remain viable while those encapsulated by resistant hemocytes are usually, but not always, killed (Borges *et al*., 1998; Goodall *et al*., 2004). As a result, we know that parasite death is not due simply to suffocation or starvation (Bayne *et al*., 2001). Killing of the parasite is widely believed to be due to the release of a variety of reactive oxygen species. Superoxide dismutase gene expression and enzyme activity have been shown to be higher in resistant strains and consequently hemocytes from resistant snails produce higher levels of H_2O_2 than do susceptible snails, H_2O_2 is known to be particularly toxic to *S. mansoni* (Bender *et al*., 2005; Loker, 2010).

Other theories exist as to where exactly the difference between a successful and unsuccessful infection lies. It has been suggested that the parasite itself is actually the determining party i.e. that the success or failure of an *S. mansoni* infection does not depend on the snail susceptibility or resistance type, but depends on the genetically ‘matched’ or ‘mismatched’ status of the host and parasite (Théron and Coustau, 2005; Bayne, 2009). The number of infecting miracidia is also thought to play an important role in the success of an infection. During experimental infections, when snails from both strains were infected with 10 miracidia the rates of successful infection for the resistant and susceptible strains were 30% and 62% respectively, but when the number of miracidia was increased to 30 both strains showed infection rates of approximately 65% (Théron and Coustau, 2005). It would appear that infection outcome is determined by a combination of host and parasite phenotype.

Both susceptible and resistant snail strains show normal and comparable reactions to other pathogens, however, one of the differences between *S. mansoni* and other intruders is its ability to actively deceive the immune system of compatible hosts. Both miracidia and sporocyst stages have been shown to present 'snail-like' antigens in a process known as 'molecular mimicry' by which the parasite is recognised as 'self' and not attacked (Yoshino and Coustau, 2011). Larval ESPs are also thought to be able to manipulate the hosts response by interfering with hemocyte functions such as motility and cell spreading (Noda and Loker, 1989; Lodes and Yoshino, 1990), phagocytosis (Noda and Loker, 1989; Connors and Yoshino, 1990; Nunez *et al*., 1997), ROS production (Connors and Yoshino, 1990) and encapsulation responses (Loker *et al*., 1992; Humphries and Yoshino, 2003).

While the exact conditions determining the success or failure of an infection remain to be established, it is clear that *S.mansoni* possesses a mechanism that, given a compatible snail, can manipulate the host genome and subsequently influence some of the processes which take place, toward a more hospitable environment (Knight *et al*., 2014).

While many studies have investigated the *B. glabrata* interaction with *S.mansoni* from a genetic perspective, the majority have tended to focus on genes known to be related to infection. Recently, Coustau *et al*., (2015) applied a non-targeted approach to survey a broader range of genes for potential immune function. This resulted in the discovery of 31 novel immune-associated transcripts which correspond to various functional groups (Coustau *et al*., 2015). This kind of work continues to increase the number of targets for investigation toward a better understanding of the molecular mechanisms which underlie the relationship and its success or failure.

Most studies on host-parasite interactions take place under artificial lab conditions, however this is a situation far removed from natural ecological systems where a host of external factors can influence the relationship (Thieltges *et al*., 2008). It is therefore easy to infer that infection levels could go up or down following a change in these conditions (Thieltges *et al.*, 2008).

An interesting example of the stark nature with which external factors can influence the parasite-host relationship is given by experiments on the effect of heat-shock on *B.glabrata* (Ittiprasert and Knight, 2012; Knight *et al*., 2015; Coustau *et al*., 2016).

Researchers found that a significant difference existed between susceptible and resistant snails regarding their level of HSP expression following parasite exposure and/or heat-shock, with the former displaying considerably elevated levels of expression when compared to the latter (Ittiprasert and Knight, 2012). Even more significant was the discovery that exposure of juvenile resistant-type snails to a mild heat shock (32^{o}C for 4 hours) resulted in a loss of resistance to *S.mansoni* infection, this alteration in resistance was also found to remain present in the progeny of generations which had previously been raised at the elevated temperature, even when these offspring snails were themselves always maintained at a normal temperature (Ittiprasert and Knight, 2012).

These results are particularly interesting given that, despite their name, many environmental stressors, aside from just heat, are capable of significantly influencing the expression of HSPs, and we are therefore led to the possible conclusion that the *B.glabrata-S.mansoni* relationship is potentially more malleable under field conditions than may have previously been thought (Mahmood *et al*., 2014). Something which is rarely considered is the impact that change in natural conditions/habitats might have on this parasite-host relationship. Furthermore, what impact might such alterations at this stage have on human infection? And what are the types of changes most likely to occur in today's world?

1.3. Does pollution affect the *B.glabrata* - *S.mansoni* relationship?

The immune systems of aquatic invertebrates and in particular molluscs, face some unique challenges. Due to the lack of an exoskeleton, as is present in arthropods, molluscs are in direct contact with the ambient environment (water). Therefore, pathogens, but also anything else in the environment (e.g. chemicals), can be taken up not only from the diet but also from the water *via* the integument and respiratory organs (Oehlmann and Schulte-Oehlmann, 2002). This is further compounded by the slow or sessile habits of molluscs and allows more time for exposure to take place.

Because Schistosome-transmitting snails occur in very particular ecological circumstances that are subject to rapid change in an increasingly human-dominated world, one of the greatest challenges is therefore to try to understand how snails will be affected by anthropogenic changes to the environment such as climate change,

increased pollution of aquatic habitats, the introduction of exotic and invasive species, construction of dams and irrigation systems and increases in human population density (Loker, 2005; Monde *et al*., 2015).

Many of the aquatic habitats in which Schistosome-transmitting snails occur have become incredibly polluted and anecdotal reports suggest that it is often necessary to remove inorganic refuse to get to the water to find snails, which are nonetheless there and even thriving (Loker, 2005). Indeed, *Biomphalaria* snails are able to tolerate poor water quality, in part because they breathe air, and will happily use plastic refuse as shelter and as a place to lay eggs. As early as 1993 pollution had been suggested as a possible cause for a change in distribution of schistosomiasis along the Nile (Abdel Wahab *et al*., 1993). Along the course of the river there has been a substantial decrease in the prevalence of *S.haematobium* and a subsequent rise in that of *S.mansoni* and this has coincided with an increase in pollution, particularly in the canals of the delta (Loker 2005; Abou-El-Naga, 2013).

In combination with the introduction of exotic strains, as has already happened in Eqypt (Kristensen *et al*., 1999), which are more susceptible to infection and may be more tolerant to pollution, there is the potential for increased transmission. The uncertainties regarding where and when these new situations will arise, with respect to snails, is further compounded by the impact of global climate change (Martens *et al*., 1997; Sutherst, 2004) and the construction of massive water development projects, such as dams, that increase vector-snail habitats across huge areas (Loker, 2005). Indeed, the Aswan Dam in Egypt created excellent habitats for the local vector snails and accordingly human infection rates rose significantly (Heyneman, 1979). Construction of other large impoundments throughout Africa (e.g. Paperna in 1969) has substantially increased Schistosome transmission, resulting in increased human disease (Lafferty and Kuris, 2005; Gryseels *et al*., 1994).

The possibilities for pollution to influence parasite-host relationships are numerous and complex, for example; chemicals may not necessarily be immunosuppressant in nature and/or may be equally, or more, toxic to the parasites thus reducing rates of exposure for some hosts. Similarly, densities of both hosts and parasites could be affected by contaminants if the contaminants affect the densities of their predators or competitors. Additionally, if contaminants reduce food resources, hosts might have

less energy to invest in immunity. Alternatively, if contaminants reduce the densities of species that prey on hosts, hosts might forage more freely thus having greater energy to invest in immunity. There are also several ways that contaminants can alter the parasite diluting effect of biodiversity. For instance, if a contaminant reduces densities of incompetent hosts, per capita parasite attack rates may be increased for the remaining hosts. This change in density might then increase parasite prevalence and thus the need to invest in immunity. A final scenario could be based on the fact that some chemicals such as xenoestrogens may cause snails to exhibit increased oviposition, thus increasing the number of hosts available for infection. (Martin *et al*., 2010; Iqbal and Sinha, 2011).

In contrast to marine bivalves, the effects of environmental pollutants on immunological defence mechanisms have been poorly investigated in aquatic gastropod molluscs, something that is quite surprising given their significance as vectors (Russo and Lagadic, 2004).

The studies which have investigated the effects of toxic pollutants on *S.mansoni* have almost exclusivley focused on the free-living stages of the parasite, typically the cercariae, and amongst the vast array of potential pollutants heavy metals seem to be disproportionately studied (Morley *et al*., 2001; Soliman, 2009). Consequently, little is known about the effects of toxic pollutants on the intra-molluscan stages of the parasite up to and including cercarial maturation and emergence (Morley *et al*., 2003). On-top of this, relatively little has been recorded regarding the levels and composition of pollutants within the water of transmission habitats, but what we do know leads us to believe that such habitats are likely subject to considerable contamination.

1.3.1. Transmission country pollution

Up to 90% of wastewater in developing countries flows untreated into rivers, lakes and coastal zones (Corcoran *et al*., 2010; World Water Assessment Programme, 2012).

In schistosomiasis endemic countries, industrial waste water and sewage containing various heavy metals, toxins, and endocrine disrupting compounds, are routinely dumped untreated directly into rivers (Iqbal and Sinha, 2011).

In Latin America, home to *B.glabrata* and *S.mansoni*, only about 15 percent of collected wastewater passes through treatment plants (with varying levels of actual treatment). In Venezuela, for example, 97 percent of the country's sewage is discharged raw into the environment (Corcoran *et al*., 2010).

In Brazil, the main transmission country for *S.mansoni* in South America, 71.5% of municipalities did not treat their sewage in 2008 (IBGE 2008). Incidentally, the regions with the lowest percentage of sewage capture and treatment (the northern and north-eastern) also appear to be among the most heavily infected with *S.mansoni* (Colley *et al*., 2014). While this does not necessarily imply a connection between sanitation and infection, and is likely due in part to the fact that much of the rest of the country is covered by dense rainforest, it does seem to suggest that *B.glabrata/S.mansoni* have a considerable risk of being exposed to aquatic pollution in these areas.

The composition of wastewater in developing countries is changing due in part to the rapidly increasing production and consumption of pharmaceutical and cosmetic products (Arnold *et al*., 2013). Figure 1.11 shows that there is typically a higher degree of variation in contaminants in the more developed transmission countries (e.g. Brazil, China and Indonesia) and also of hormones, possibly indicating higher levels of pharmaceutical use. For example, in Brazil studies on freshwater supplying Sao Luis have found considerable levels of the industrial chemical BPA, between 1.11 and 3.61 μg per Litre (Melo and Brito, 2013).

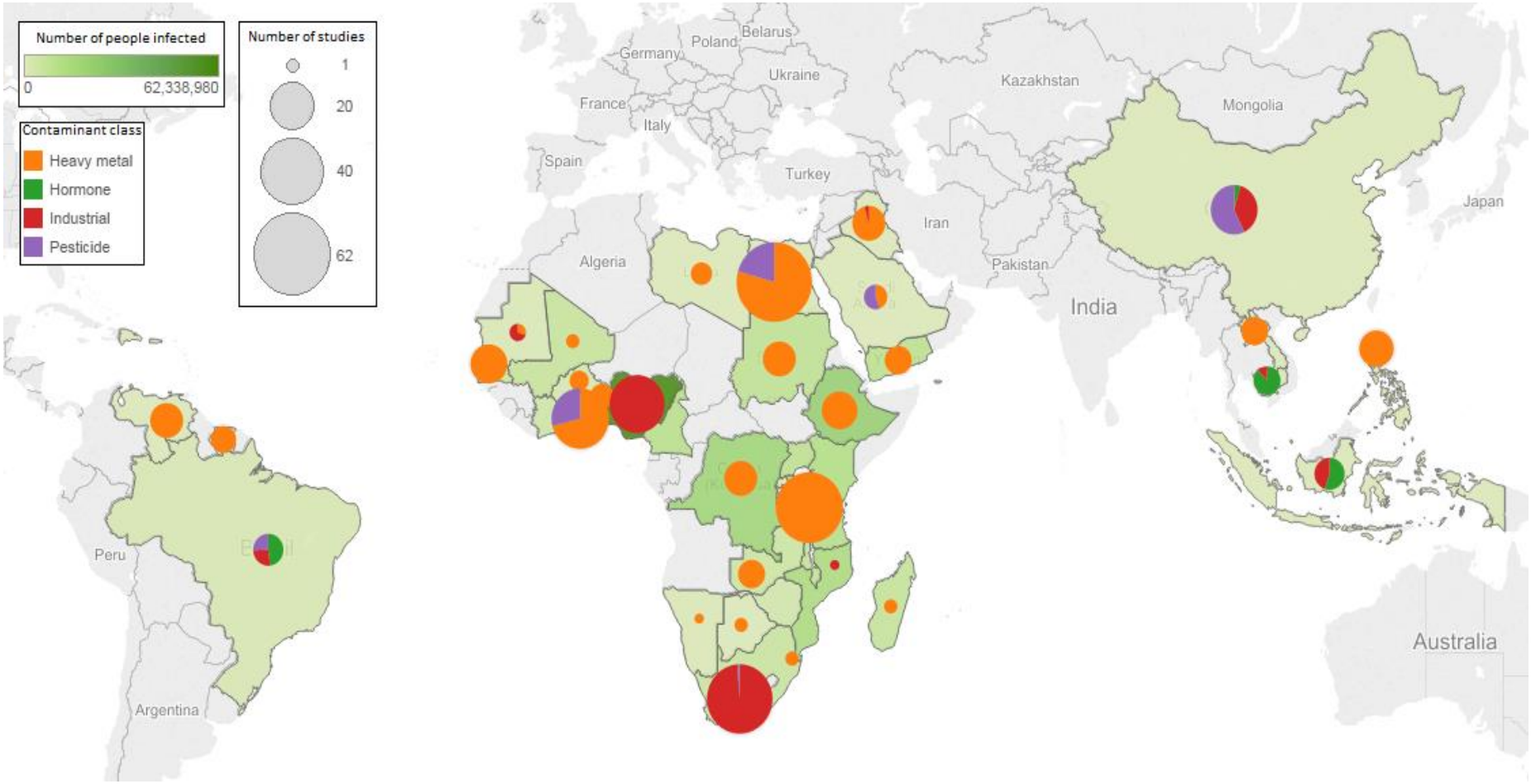


Figure 1.11. Pollutant classes and levels present in freshwater bodies of *S.mansoni* transmission countries. *The green shading represents the number of people infected based on praziquantel treatment data (WHO, 2013); colour of pie charts represents chemical class; proportion of the pie represents the average concentration of chemicals (µg/L, square-root transformed) in various freshwater bodies across multiple studies; overall size of each pie represents the total number of studies found for that country.*

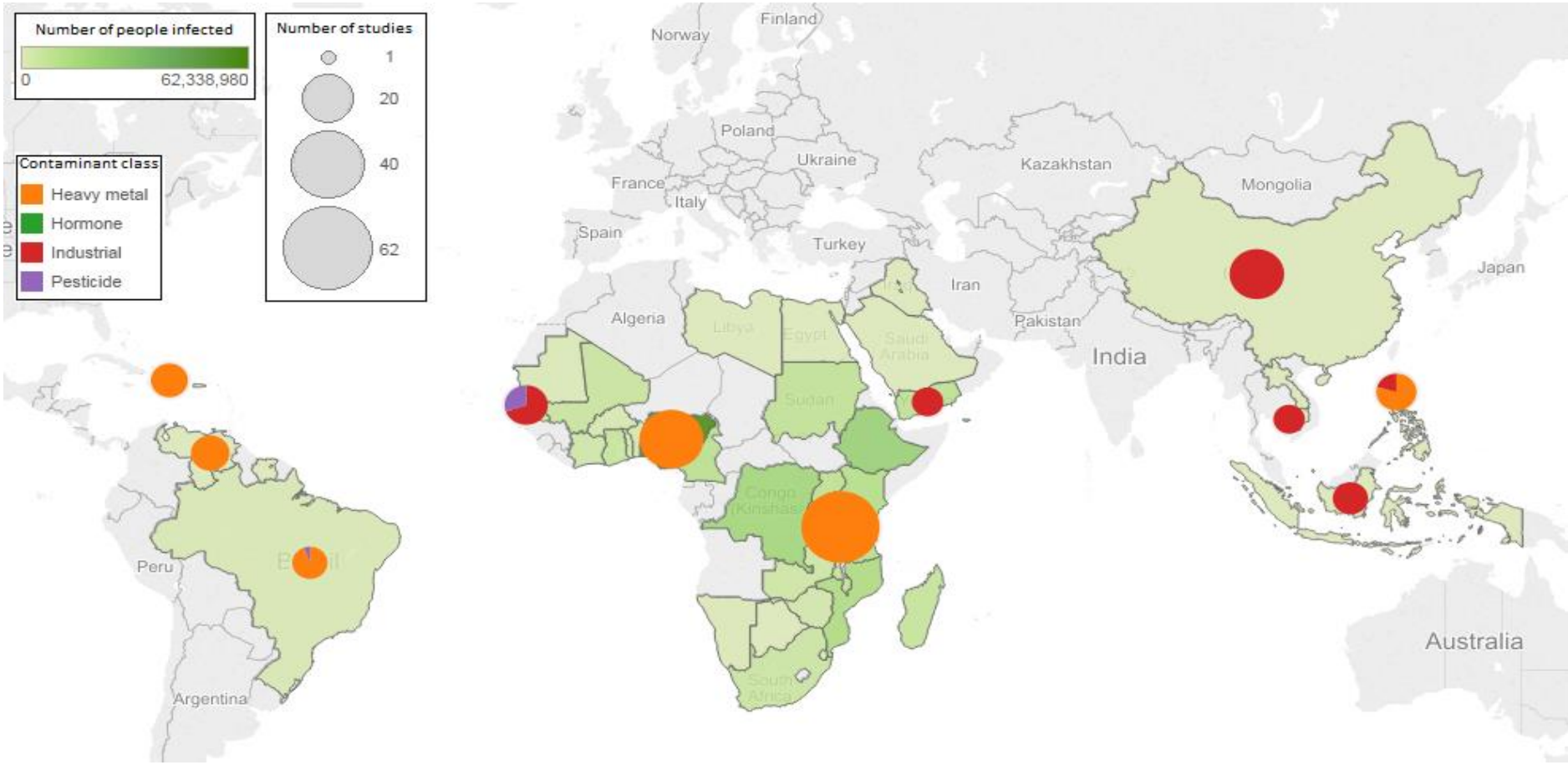


Figure 1.12. Pollutant classes and levels present in aquatic molluscs from *S.mansoni* transmission countries. *The green shading represents the number of people infected based on praziquantel treatment data (WHO, 2013), the colour of pie charts represents chemical class; proportion of the pie represents the average concentration of chemicals (µg/g, square-root transformed) in various mollusc species across multiple studies; overall size of each pie represents the total number of studies found for that country.*

Of course, the problem of pollution in the environment is a much broader issue than in relation to *B.glabrata/S.mansoni* alone. The types and routes of pollutants into global ecosystems are almost innumerable and thus tend to be tackled individually or in classes. Due to the impossible task of considering all forms of environmental pollution and their subsequent effects, as well as the considerable importance posed by Schistosomiais, it seems that the relative potential for aquatic pollutants to alter transmission of this disease is a worthy area, especially of study given the extremely small amount of existing information.

While it is not feasible to tackle a much broader problem relating to environmental pollution, it is still important to understand the general area within which we are working and this is an area of study known as ecotoxicology. Furthermore, by broadening our understanding of the field, as we have already done for Schistosomiasis, its causative organisms and their biology, we can see exactly how and where the concept of *B.glabrata/S.mansoni* habitat pollution affecting the potential for transmission fits with the wider existing knowledge. This, combined with our understanding of Schistosomiasis, will help lead us to the ultimate aim which is the formulation and understanding of a hypothesis based on these ideas.

1.3.1.2. Ecotoxicology; pollutants in the environment

A range of anthropogenic activities including industrial emissions, sewage disposal, biocide application and industrial accidents are introducing large quantities of 'foreign' chemicals into the environment. A considerable body of evidence has accrued demonstrating that these so called 'xenobiotics' can severely impact ecosystems even at low doses and this can occur in the short, medium and long term. The general consensus is that many of these chemicals may directly, or indirectly, cause detrimental effects to the main physiological systems of many different species (Boudou and Ribeyre, 1997; Fournier *et al.*, 2000). Some of the best known examples include the biomagnification of pharmaceutical products and pesticides such as *p,p*-DDE and diclofenac, which have been shown to cause egg-shell thinning in gulls and dramatic declines in vulture population respectively (Connon *et al.*, 2012).

Although naturally occurring chemicals like phytoestrogens can also be considered xenobiotics if introduced to foreign ecosystems, researchers tend to draw a distinction between these chemicals and those which are synthetic. With most in-organic chemicals it is desirable that they do not exist in the environment at all, however typically we must concede that some of these chemicals will inevitably remain and thus the task is to monitor them and ensure that they do not reach a harmful level. We are then left with the obvious question of what warrants 'harmful'? And for which species? This is something which continues to be an important area of research (Walker, 2012). Of central importance is the relationship between the quantity of chemical to which an organism is exposed and the nature and degree of subsequent harm, for example, determining selectivity ratios between beneficial organisms and non-beneficial organisms when applying pesticides (Walker, 2012).

The discipline of 'ecotoxicology', a hybrid of approaches from ecology and toxicology, was born as a result of these growing concerns and a need to study the effects (Relyea and Hoverman, 2006). Since contaminants can affect different levels of biological organization, ecotoxicological studies may focus on toxic effects at a smaller scale than suggested by the 'ecology' aspect, such as individual animals, physiological mechanisms within cells and even DNA responses (Preston, 2002).

Of all ecosystems, it is those that are primarily aquatic that are subject to the most diverse range of chemical contaminants since the routes by which foreign chemicals can enter them are so numerous. Indeed, Pritchard *et al.*, (1993) refer to the waters of our planet as the ultimate sink for chemical pollutants. Chemicals can enter aquatic ecosystems directly from municipal and industrial wastewater effluents and indirectly from airborne deposition, as well as runoff from urban and agricultural areas (Connon *et al*., 2012). Furthermore, contaminants in water are easily dispersed *via* the movement of the water and by food-chain transfer (Gagnaire *et al.*, 2006). Consequently, there is an increasing interest in toxicology related to effects on the aquatic environment, not least because it constitutes 70% of the surface of the globe, is necessary for all life and is a valuable source of commerce (e.g. fishing, petrochemicals, minerals, energy), (Mothersill and Austin, 2003).

The effects of pollutant chemicals in the aquatic environment at large quantities (e.g. oil spills) are readily observable and draw much attention from the public. However, subtle or delayed effects of considerably smaller quantities of compounds are more difficult to determine but may potentially be equally damaging over time (Mothersill and Austin, 2003). With the advance of analytical techniques like gas chromatography, thin-layer chromatography and atomic absorption allowing us to study in more detail, it has become clear that acute toxicity is not the only concern and that low-level exposure can lead to accumulation of certain chemicals resulting in wide ranging effects (Pritchard, 1993; Walker, 2012).

Within the field of ecotoxicology the studying of aquatic xenobiotics, their differing concentrations, dispersion modelling, combinations and the relative sensitivity of a species, communities or ecosystems, and the intensity, timing and mechanisms of exposure are all part of the aims of aquatic toxicology (Connon *et al*., 2012).

1.3.1.2.1. Aquatic ecotoxicology

Depending on the objectives/scope of an investigation, studies can be performed under standardized conditions in laboratories using cell systems (*in vitro*), whole organisms (*in vivo*), small-scale artificial communities (mesocosms) or in field studies, where resident organisms are sampled and assessed.

From the view of the aquatic toxicologist, the problem of predicting adverse effects is made more difficult by the fact that chemical contaminants in the environment seldom occur in isolation, but are present as a complex mixture. Consequently, cause and effect relationships are often difficult to establish (Lowe, 1995). *In vitro* tests help with such dilemmas as they are capable of high throughput applications, allowing the fast screening of many different chemicals, individually and as mixtures, something that would be impossible on a much larger scale. Chemicals of interest can then be selected and used for *in vivo* testing allowing the *in vitro* tests to be linked with effects at the population level (Connon *et al.*, 2012).

In vivo bioassays are tests in which whole living organisms are exposed to samples (water, sediment) spiked with a chemical, or extract, of interest. Standardized bioassays rely on measuring responses in model species (e.g. Zebra fish) which may

not be representative of all species, but, allow for the quantification of chemical-caused toxic effects that are separated from environmental confounders. Such bioassays routinely form the basis of legal limits when testing products or water discharge quality (Connon *et al.*, 2012). The response which is being measured is often referred to as a 'biomarker' which is defined as a biological response that can be measured at the level of the whole organism, in tissue samples, body fluids and even at the genetic level, to signal exposure to, or adverse effects of, anthropogenic chemicals (Galloway and Depledge, 2001; Walker, 2012).

The development of toxicogenomics and the associated ability to perform high-throughput screening has significantly enhanced the ability to identify important molecular events associated with toxicity (Beyer *et al*., 2014). Toxicogenomics is the application of 'omics' approaches toward identifying the effect of toxic chemicals at the genetic level, this includes the measurement of genomic changes (i.e. epigenetics), the transcription of genetic information to mRNA (transcriptomics), the translation of mRNA to protein synthesis (proteomics) and metabolic activities and their related products (metabolomics), (Beyer *et al*., 2014).

Studies in aquatic toxicology are often based on freshwater ecosystems. As they are typically easier to study, being more constrained geographically, with clearly identifiable upstream and downstream areas and there are often clear point sources of contamination. In situations where point sources are not obvious, it is generally possible to identify which human activities are likely to be affecting a given body of water (Blanar *et al.*, 2009).

In urban areas sewage effluents are the main source of anthropogenic chemicals discharged into the freshwater environment (Magdeburg *et al*., 2012). Domestic wastes are discharged mainly into sewage systems and industrial wastes are discharged either into the sewage system or directly into surface waters. The 'quality' (defined as the nature and concentration of chemicals present) of the sewage that is discharged into surface waters depends on the quality of the raw sewage received by the sewage works and also the level of treatment that the sewage is subjected to within the works (Walker, 2012). Even with the best practices in sewage treatment such waste waters commonly contain complex mixtures of thousands of synthetic chemicals and their biotransformation products, including

flame retardants (e.g. PBDEs), plasticizers (e.g. BPA, phthalates) and pharmaceuticals (e.g. EE2, carbamazepine) (Magdeburg *et al*., 2012). As well as chemicals contained within sewage effluents, other chemicals such as pesticides have the potential to enter aquatic habitats from direct application, terrestrial runoff or wind-borne drift (Relyea and Hoverman, 2006).

The potential of some of the many substances found in sewage and in the receiving environment to disrupt physiological processes in wildlife, even at low (ng/L) levels, has raised concerns about the effects of such contamination on animal and human health (Hoeger *et al.,* 2004). One of the best studied examples of this is that of endocrine disrupting chemicals (EDCs).

For many years, toxicologists had relied on the presumption that higher doses of a chemical cause greater harm than low doses. While true of many chemicals, numerous studies have shown that EDCs often contradict this rule and display non-monotonic dose responses, whereby effects are seen at very low and very high doses, but less so in the middle of the range (Schug *et al.,* 2011). Welshons *et al.,* (2003) suggest a possible explanation in that hormones like 17ß-estradiol work at extremely low concentration levels, far beneath the levels at which all hormone receptors would be bound. Once all receptors are bound, further increases in the natural hormone can't increase the response of the system, at least *via* the interaction of the hormone and its receptor. The responses seen at high levels may then be due to more acute toxicity (Welshons *et al.,* 2003).

Endocrine disrupting chemicals (EDCs) include both natural and synthetic steroid estrogens, as well as xenoestrogens (those which happen to mimic the structure of endogenous estrogen), such as alkylphenols and alkylphenol ethoxylates, PCBs, dioxins and various pesticides. These chemicals act by selectively binding to hormone receptors to generate (agonists) or block (antagonists) hormone-mediated responses, as well as interfering with hormone synthesis, metabolism and excretion (Jobling and Tyler, 2003). There is now considerable evidence from both laboratory and field studies to indicate that EDC exposure is at least partly responsible for disruption in the reproduction and development of certain species (Canesi *et al.,* 2005).

In wildlife species, unlike humans (where findings typically provoke controversy), it is clear that there exist examples of male and female reproductive digenesis and thyroid hormone disruption in certain groups, most notably fish. Probably the most well documented examples of endocrine disruption in fish come from studies of Wild Roach (*Rutilus rutilus)* and Gudgeon (*Gobio gobio)*, living in British rivers, where the presence of vitellogenin (VTG), an oestrogen–dependent, and normally female-specific protein, in the blood of male fish as well as intersexuality (the presence of ovotestis) are widespread (Jobling and Tyler, 2003). In most cases, observations have been associated with exposure to effluent from sewage treatment works (STWs), where steroidal oestrogens play a major role in causing ED (Desbrow *et al.*, 1998).

A considerable body of work also points to very clear evidence of feminisation of secondary sexual characteristics, altered sex hormone concentrations and intersex in amphibians (Desbrow *et al.*, 1998; Hayes *et al.*, 2002; Bögi *et al.*, 2003; Mosconi *et al.*, 2005).

In contrast, little is known about the fundamental endocrinology of many invertebrate species and so there is currently limited capacity to study endocrine disruption or its potential in these groups (EEA, 2012). It is believed that endocrine-signalling mechanisms exist in invertebrates as oestrogen receptor orthologous and have been reported in certain molluscs, although their exact function remains to be determined (Canesi *et al.*, 2007b; Keay *et al.*, 2009; Baker *et al.*, 2011; Benstead *et al.*, 2011; Gust *et al.*, 2013b).

It is interesting to note that many EDCs are also implicated in disruption of normal immune functioning in various species. The interactions between the immune and endocrine systems means that endocrine disruption and immunotoxicity can, and do, occur simultaneously (Gagné *et al.*, 2008; Jobling and Tyler, 2003).

Endocrine disrupting chemicals are by no means the only class which can have significant effects on the immune systems of numerous aquatic animals across a range of species, classes and phyla. While there is significant interaction between the endocrine and immune systems and alterations in the immune system can be a consequence of endocrine disruption, EDCs can be directly toxic to the immune system as can a whole host of different chemicals from several classes, not least

pesticides, which, in the case of invertebrates, are often designed for this very purpose.

1.3.1.2.2. Immunotoxicology

Many of the chemicals introduced into the environment as a result of industrial or agricultural activity have been implicated in ecotoxicological effects mediated via immunotoxic mechanisms and there is a growing body of evidence to suggest that such contaminants can adversely affect immune function in both humans and wildlife (Galloway and Depledge, 2001).

Immunotoxicity refers to the fact that the immune function of many organisms can, like that of other physiological/homeostatic systems, be altered by exposure to sub-lethal concentrations of environmental contaminants, potentially leading to enhanced susceptibility to, and progression of, infectious diseases and cancerous disorders (Blaise *et al*., 2002). The situation is further complicated as in reality immunosuppression may not be the only response xenobiotics can induce, immune stimulation, increase in disease resistance, decrease in disease virulence and auto-immunity are also potential outcomes in exposed organisms (Gagnaire *et al.,* 2007).

As in all immunotoxicological studies, alterations in the response of selected immune parameters, which will be discussed later, cannot be rated as indicative of overall immune competence without specific evidence of increased probability of infection (Hoeger *et al.*, 2004), (Table 1.3).

Over the past decades, the emergence of infectious diseases related to anthropogenic chemical contamination has been reported in many aquatic species and disease outbreaks are, on the whole, also believed to have increased (Harvell *et al*., 1999). Some examples of such findings across various classes can be seen in Table 1.3.

Table 1.3. An example of selected studies which focused on the effects of various xenobiotics on the outcome of infectious diseases in different aquatic species.

Species	**Pollutant**	**Outcome**	**Reference**
Phocoena phocoena (harbour porpoise)	PCB	Increase in fatal infectious diseases	Gagnaire *et al*., (2007)
Rana sylvatica (Wood frog, tadpoles)	Pesticides	Increase in trematode infections and associated morphological deformities of limbs.	Kiesecker, (2002)
Various oysters species along the Gulf of Mexico	Selenium	Decrease in prevalence of pathogen *P.marinus*	Wilson *et al*., (1992)
Mytilus trossulus (Mussel)	Untreated sewage	Increase in prevalence of trematodes	Moles and Hale, (2003)
Clarias gariepinus (African Catfish	Untreated sewage	Decreased nematode prevalence	Madanire-Moyo and Barson, (2010)

The precise mechanisms through which different chemicals can cause alterations in immune function vary according to the type of chemical, the affected species and often also the nature of the pathogens which naturally infect it.

For example, Khan (1990) showed that oil pollution led to an overall increase in the intensity of gill-ciliate infections in fish. The proposed mechanism by which oil increased susceptibility was through toxicity to the fish's ability to produce mucus, a first line in protection against gill parasites (Khan 1990; Lafferty, 1999).

Probably the best documented mechanism of pollutant effects on the immune system is direct toxicity to its components. Isolating immune cells, exposing them to particular contaminants, and quantifying survival and function has been an effective way to demonstrate direct toxicity of contaminants (Martin *et al*., 2010). Apart from simply being a substitute for vertebrates, aquatic invertebrates are an integral part of all aquatic biomes and biotypes and the effects of toxicants upon them could alter the structure and functioning of aquatic ecosystems (Rickwood and Galloway, 2004).

For example, several studies have investigated risks to birds feeding on invertebrates exposed to sewage effluent from trickling filters. These have indicated that invertebrates living on, and emerging from, such locations have the potential to bioaccumulate/bioconcentrate certain pharmaceutical EDCs. This is a potential problem further up the food chain since studies on adult male starlings (*Sturnus vulgaris*) experimentally exposed to environmentally relevant levels of various EDCs displayed altered immune function and changed development that affected behaviours such as singing; reduced growth and depressed immunocompetence were also observed in the nestlings (Shore *et al*., 2014).

1.3.1.2.2.1. Immunotoxicology in Invertebrates

Invertebrate models are necessary in many areas of biomedical research, but it seems that they often go unheeded in ecotoxicology, despite constituting 95% of animal species and being key components of all ecosystems (Berger, 2010; Pinder *et al*., 1999; Galloway and Depledge, 2001). When invertebrates like *Daphnia pulex* are used in research there is a tendency to extrapolate responses to other species and phyla. This approach fails to take account of the diversity of physiological functions that render different phyla vulnerable to different types of toxicants at different stages in their lifecycles. Clearly, a stronger scientific basis for ecotoxicology depends upon understanding the range of susceptibility to pollutants that occurs among invertebrate species (Galloway and Depledge, 2001).

In recent years more studies have begun to focus on other invertebrate phyla, for use in immonotoxicological studies, although valid and interesting results have arisen the scope remains relatively restricted, particularly to bivalve molluscs when considering aquatic studies (Oehlmann and Schulte-Oehlmann, 2002). Canesi *et al*., (2004) showed that the human sex hormone estradiol (E2), which is present in waters receiving domestic effluent, induced rapid changes in *Mytilus galloprovincialis* cell shape, lysosomal membrane destabilization and release of hydrolytic enzymes *in vitro*. Low-level exposure to PCP (a pesticide residue and common contaminant of aquatic environments) altered transcription of genes involved in cellular metabolic activities including the up-regulation of hormonal genes in bloodworms (*Chironomus riparius* larvae) (Morales *et al.*, 2014).

In contrast to the complexity of the vertebrate immune system, the comparatively simpler structure of the invertebrate immune system makes it a potentially more sensitive, but also more accessible, means of monitoring the effects of environmental contaminants and the complex interactions which ultimately underpin host resistance and disease transmission (Galloway and Depledge, 2001). In order to understand the effects that any chemical pollutants might have on immune function and disease resistance we must first understand and identify the components of the invertebrate immune system that may serve as biomarkers.

1.3.1.2.2.2. Immunotoxicology in molluscs

Under normal conditions the immune system of molluscs maintains an efficient protection against various pathogens, as demonstrated by the great size and diversity of the phylum. However, its efficiency may be weakened by external factors, including the rapid rise of anthropogenic chemical contaminants in water, this is something which normal evolutionary adaptation has not had sufficient time to address (Auffret *et al.*, 2002).

Compared with vertebrates and some other invertebrate groups like arthropods, molluscs exhibit only a limited ability to excrete pollutants directly via their kidneys or other excretory organs, to metabolise organic chemicals and to physiologically inactivate them. As a consequence of this, and due to their relatively immobile life strategies and direct exposure to water, molluscs often attain higher bioconcentration factors for many toxicants than do other groups (Oehlmann and Schulte-Oehlmann, 2002). For this reason, it has been suggested that environmental pollutants may exhibit negative impacts on mollusc immune systems at lower concentrations than may be the case for other invertebrates or vertebrates. Indeed, a substantial body of data has emerged to support this view (Oehlmann and Schulte-Oehlmann, 2002).

For these reasons certain molluscs have been used as indicator species. These species, which are typically amongst the first to experience detectable signs of environmental changes, like pollution, act as an early warning for the rest of the ecosystem.

There are a number of other reasons that molluscs have been used in immunotoxicological studies. Unlike vertebrates, molluscs are a non-controversial organism for use in research and compared to other invertebrates they tend to be relatively large and easy to handle and culture (Oehlmann and Schulte-oehlmann, 2002). Another reason for the interest in using molluscs in ecotoxicology research is that they represent a very commercially valuable group. Edible species of mollusc (e.g. mussels, scallops and oysters) are particularly well studied due to their economic importance around the world and because of their potential susceptibility to pollutants and/or disease (Gagnaire *et al*., 2003).

1.3.1.2.2.3. Immunotoxicology in commercial mollusc species

Bivalves constantly filter large volumes of water and thus are at an increased risk of bioaccumulating environmental contaminants within their tissues (Gagnaire *et al*., 2003). Table 1.4 summarizes some of the many studies which have investigated the relationship between innate immunity in bivalves and certain environmental pollutants. Research on commercially valuable species of gastropod mollusc has reported xenobiotic-induced negative impacts on their immune systems resulting in increased disease susceptibility (Gopalakrishnan *et al*., 2011).

Global demand for seafood, including molluscs, continues to grow (FAO, 2009). However, disease continues to be a major financial constraint to growth of mollusc culture (Jones, 2011). The productivity of oyster aquaculture in the United States is often seriously diminished by the pathogens *Perkinsus marinus* and *Haplosporidium nelsoni* (Goedken and De Guise, 2004).

The European flat oyster, *Ostrea edulis*, has been severely attacked for two decades by the intra-hemocytic parasite, *Bonamia ostreae* (Auffret *et al*., 2002).

Many of the major diseases of commercial mollusc species are parasites and many of their habitats are often highly polluted. Therefore, investigation into the ways that contamination can impact the mollusc immune response against parasites is needed.

Table 1.4. Compilation of various studies on the immunotoxic effects of different pollutants on commercial mollusc species. *The classes of xenobiotics included in these studies include metals, pharmaceuticals, industrial bi-products and natural hormones*. Table adapted from Girón-pérez, (2010).

Species	Xenobiotic	Effect on immune response	Reference
Ostrea edullis	Cadmium	Hemocyte count↑, cell membrane potential↓	Auffret et al., (2002)
Ostrea edullis	Cadmium/copper	Phagocytic activity↓	Auffret et al., (2002)
Crassostrea virginica	Cadmium	Methalothionein↑, ROS↑	Butler *et al*., (2000)
Mytilus galloprovincialis	Cadmium and copper	Hemocyte viability↓, phagocytosis↓	Gomez-Mandikute *et al*., (2003)
Elliptio complanata	Copper	Hemocyte count↓, superoxide anion↓	Gagne *et al*., (2008)
Mytilus edulis	Copper	Phagocytosis↑	Parry *et al*., (2004)
Mytilus galloprovincialis	Nonylphenol, monoethoxilate, carboxylate, 17α-ethynylestradiol	Lysosomal enzyme release↑, Phagocytosis↑↓	Gagne *et al*., (2008)
Corbicula fluminea	17β-estradiol	Phagocytosis↓	Canesi *et al*., (2007)
Elliptio complanata	Benzafibrinate, gembibrizol, trimetophin	Phagocytosis↓	Gagne *et al*., (2006)
Elliptio complanata	Novobiocin, morphin	Phagocytosis↑	Gagne *et al*., (2006)
Elliptio complanata	Zulfamethazole, novobiocin, gemfibricil, benzafibrate, carbamazepine	Esterase activity↓	Gagne *et al*., (2006)
Elliptio complanata	Oxytetracycline, novobiocine, naproxen	Cell adherence↓	Gagne *et al*., (2006)
Elliptio complanata	Gemfibrozil, bezafibrate	Cell adherence↑	Gagne *et al*., (2006)
Elliptio complanata	Novobiocine, sulfapyridine	Lipoperoxidation↑	Gagne *et al*., (2006)
Elliptio complanata	Coprostanol, naproxen	Lipoperoxidation↓	Gagne *et al*., (2006)
Crassostrea gigas	Benzo[a]pyrene, phenanthrene	Granulocyte %↑, Cell viability↑, Esterase, Lysosome-positive cells↓	Gagnaire *et al*., (2006)
Mytilus galloprovincialis	Benzo[a]pyrene	Hemocyte viability↓	Gomez-Mandikute *et al*., (2003)
Chamelea gallina	Benzo[a]pyrene	Lysozyme activity↓, Phagocytosis↓, cell adherence↓	Matozzo *et al*., (2009)
Cerastodema edule	Phenanthrene	Hemocyte viability↓, phagocytic cells↓, superoxide generation↓	Wootton *et al*., (2003)
Pecten maximus	Phenanthrene	Hemocyte count↑, cell membrane stability↓, phagocytosis↓	Hannam *et al*., (2010)
Crassostrea gigas	PCB 77	Lysosome-positive cells↓	Gagnaire *et al*., (2006)
Crassostrea gigas	2,4D	Cell viability↓	Gagnaire *et al*., (2006)
Crassostrea gigas	Paroxon	% Esterase and lysosome-positive cells↓	Gagnaire *et al*., (2006)
Crassostrea gigas	Chlorothalonil	ROS-positive cells↑, cell viability↑, granulocyte %↑	Gagnaire *et al*., (2007)
Crassostrea gigas	Atrazine, glyphosate, alachlor, methalaclor, fosethyl-aluminium, terbuthilazine, diuron, carbaryl	Phagocytosis↓, cell viability↓, ROS↑, genes related to immune-response↓, susceptability to bacterial challenge↑	Gomez-Mandikute *et al*., (2003)

1.3.1.2.2.4. Parasitism and immunotoxicology in molluscs

The overall impacts that anthropogenic pollution has on the organisms that live in the affected areas are as yet poorly understood, but disease emergence is known to be more common in urban areas than all other habitat types. Much of the association between urban areas and increased disease emergence is related to the higher availability of vector breeding sites, climate moderation, or high densities of some hosts, but some suggest that it may also be attributable to variation in host immune functions (Martin *et al*., 2010).

The parasitic lifestyle is widespread in the animal and plant kingdoms. It is widely accepted that more than a half of all existing animal species are temporarily or permanently parasitic and parasitic organisms can be found in nearly every environment, ranging from the poles to the tropics. (Pietrock and Marcogliese, 2003).

Current research on the effect of pollutants on parasite-host interactions has focused on amphibians due to the global amphibian decline. The observation that pesticides can have both positive and negative effects on this relationship supports similar findings in other phyla (Relyea and Hoverman, 2006).

The effects of pollution on parasitism in molluscs are variable and may be positive or negative. An increase in parasitism may be considered likely if the pollutant inhibits the immune system of the host rather than the parasite. For example, Auffret and Oubella, (1997) exposed *C. gigas* oysters to xenobiotics which had been identified in a polluted estuary. In response to some chemicals they observed changes in hemocyte aggregation behaviour and speculated that the aggregation activity of these cells may be altered by stress induced from the environmental contamination. Nodule formation has also been shown to be affected in molluscs as a consequence of pollutant exposure (De Vico and Carella, 2012). Since the aggregation and nodule formation behaviours are important in the mollusc response to parasites this could implicate pollution as one of the factors responsible for losses in commercial species. In contrast, pollution can also negatively affect parasitism if parasites are more susceptible to the particular pollutant than their hosts (Sures, 2006).

1.3.1.2.2.5. Molluscs and parasites as indicators of ecosystem health and beyond

Virtually all free-living organisms are hosts to a variety of parasites. A healthy ecosystem therefore cannot be considered disease free; the presence/absence of parasites reveals much about the ecology, such as interactions in the food web, biodiversity and environmental stresses (Madanire-Moyo and Barson, 2010). For these reasons, several authors have advocated the inclusion of parasites in biological monitoring studies since their presence or absence can be used to make conclusions about the impact of certain pollutants on a habitat (Table 1.5), (Blanar *et al*., 2009).

Molluscs have been successfully used to obtain information on the quality of terrestrial, marine and freshwater ecosystems and to quantify the exposure to, and effects of, contaminants in their environment. This is particularly true for the two most diverse classes of molluscs, gastropods and bivalves (Oehlmann and Schulte-Oehlmann, 2002). Indeed, it is suggested that due to the important roles molluscs play in ecosystem function a pollutant that affects a mollusc population will also likely exhibit a negative impact for the entire ecosystem (Markert *et al*., 2003; Martin *et al*., 2010). By extension, since parasites play an important role in mollusc populations one would logically infer that disruption of this relationship might have broad ecological repercussions.

Historically, parasitism has been primarily studied in molluscs of commercial interest, such as marine bivalves and there are relatively few investigations examining the interaction between ecotoxicology and parasitology, particularly in freshwater ecosystems (Minguez *et al*., 2009). This is surprising considering the fact that the relationship between aquatic molluscs and their parasites is of significant importance to man and not only for commercial purposes. Molluscs act as vectors for numerous medically important parasites and diseases. In fact, some 350 snail species are estimated to be of possible medical or veterinary significance (Oehlmann and Schulte-Oehlmann, 2002; WHO, 1995).

Table 1.5. A meta-analysis of reported associations between anthropomorphic environmental impacts and parasite abundance. Adapted from Lafferty (1997).

	Net effect on abundance of parasite species			
	Positive	**Neutral**	**Negative**	***P****
Impact type				
Heavy metals	2	0	13	<0.05
Disturbance	3	0	15	<0.05
Acid precipitation	1	2	3	NS
Sewage-sludge	3	7	6	NS
Industrial effluent	15	6	22	NS
Crude oil	6	1	5	NS
Pulp-mill effluent	14	3	9	NS
Thermal effluent	9	1	5	NS
Eutrophication	11	0	1	<0.05
Parasite group				
Digenea	13	1	31	<0.05
Acanthocephala	5	2	11	NS
Cestoda	6	4	11	NS
Monogenea	12	5	9	NS
Nematoda	7	2	4	NS
Ciliophora	11	0	0	<0.05
Other groups	10	6	13	NS

P is based on the two-tailed sign test with Bonferroni correction. Comparisons were drawn from the literature

1.4. The hypothesis

Schistosomiasis research and that of ecotoxicology over-lap, due primarily to the snail vector which inhabits waters known to contain many anthropogenic xenobiotic-chemicals. As we have seen from the preceding review of the literature, a host of environmental contaminants have been shown to impact the immune systems of many species of aquatic animal and in many cases this can be related to increased susceptibility and prevalence of infectious diseases, including parasites (Table 1.3). While there is a considerable body of data to demonstrate such effects in aquatic molluscs, the majority have focused on species of commercial interest and consequently there is a considerable gap in knowledge as to which chemicals may affect the mollusc vector of *S.mansoni* and the ways in which such effects might alter its relationship with the parasite.

The aim of this thesis was to test the hypothesis that the host-parasite relationship is affected by pollution. More specifically, I aimed to test this hypothesis using the host-parasite model of *Biomphalaria glabrata* and *Schistosoma mansoni,* due to the lack of previous research on these organisms in an ecotoxicological context. For the purpose of my studies 'pollution' took the form of several known environmental contaminants, selected based primarily on their prevalence and diversity of chemical class, described in Chapter 3.

1.4.1. The importance of method development in testing the hypothesis

The already considerable challenges in the field of ecotoxicology are even greater in tropical countries since most of these countries are in a developing status, with relatively scarce financial resources and a lack of infrastructure for advanced pollution control and research (Kwok *et al.*, 2007). Furthermore, despite having substantially higher biodiversity, it is well known that tropical/sub-tropical ecosystems are less studied than temperate ecosystems (Krull and Barros, 2012). This is also one of the ironies of schistosomiasis research; much of the necessary background information and literature, and many of the new tools and techniques, are far more readily available in developed countries that are far from endemic areas (Loker, 2005).

Ecotoxicology as a discipline is still relatively young and therefore we have much scope to develop new approaches and techniques even as individuals, something which is less common in older, more saturated fields such as cancer research. Therefore, this thesis will devote considerable attention to method development and validation. The various approaches described in the preceding chapters were borne out of the need to adapt or create techniques in an area of research that remains relatively niche. Nevertheless, the outputs of this research, on the whole, are transferable to many other aspects of biological science.

In his 2005 paper titled 'Research on the Molluscan Intermediate Hosts for Schistosomiasis: What are the Priorities?' Eric Loker suggests that it is necessary to "turn inward" and gain a greater appreciation of the basic biology of snails, trematode larval stages and their interactions, and that for this, a modern toolkit is required (Loker, 2005). Loker goes on to say that "with respect to controlling schistosomiasis at the level of the molluscan host, the true enemy is not the snail that is hosting the parasite, but the schistosome sporocysts that colonize the snail" (Loker, 2005). Indeed, much attention is focused on combating the disease at the human stage when in reality, impacts to any stage of the parasite will have a similar ultimate outcome since, by definition, the life cycle is cyclical - each point relies on the previous.

More investigations are currently needed on the mechanisms behind the effects of man-made changes on host-parasite interactions and particularly in those species of medical, rather than just commercial, importance (Budria and Candolin, 2014).

The following chapters will attempt to shed further light on the effects that 'pollution' has on the relationship between *S.mansoni* and *B.glabrata*. Focus will be on the key immune functions of *B.glabrata* and their response to selected contaminants, with the aim of establishing which, if any, aspects of the snail immune system are being affected, as well as the possible mechanisms by which this might happen. As the main effector of the snails' immune response to the parasite (and other pathogens) hemocytes will be used as the main units of interest/exposure in a series of *in vitro* experiments. Hemocyte parameters identified as being important in the snails' response to intra-molluscan stages of the parasite (and therefore to transmission) are part of an interconnected chain of events which often occur in

unison. While part of the same process, these parameters will be separated into functional categories so as to investigate the response in more detail at a cellular level. For the most part, the methods used will be novel adaptations of existing techniques, which were needed in order to answer my specific questions and in other cases I will describe in greater detail those methods that are sufficiently novel (and/or have wider applications) to warrant further description.

In order to investigate the hypothesis in a more 'environmentally relevant' context, I also performed an *in vivo* developmental exposure experiment on live snails. Here I attempt to link exposure to contaminants directly to potential alterations in parasite transmission by infecting exposed and unexposed snails and quantifying the rates of the human infective stage (cercariae) that are ultimately produced.

While molecular work would be extremely useful in helping address the hypothesis, the basis for any such work (i.e. the effect of pollutant chemicals on the immune system of *B.glabrata* and the interaction between *B.glabrata/S.mansoni* in the presence of such chemicals) has not previously been established and existing methods from which to base such research are missing. Significant effort was therefore devoted to developing and adapting methods for this purpose.

It is extremely difficult for any work to prove/disprove a hypothesis when based on information gathered from a single focus of study and therefore, as demonstrated by Figure 1.13, detailed investigation into mechanistic aspects (e.g. 'omics') may have limited value when considering ecosystem-level implications. The work that follows is a beginning to what promises to be a very broad topic.

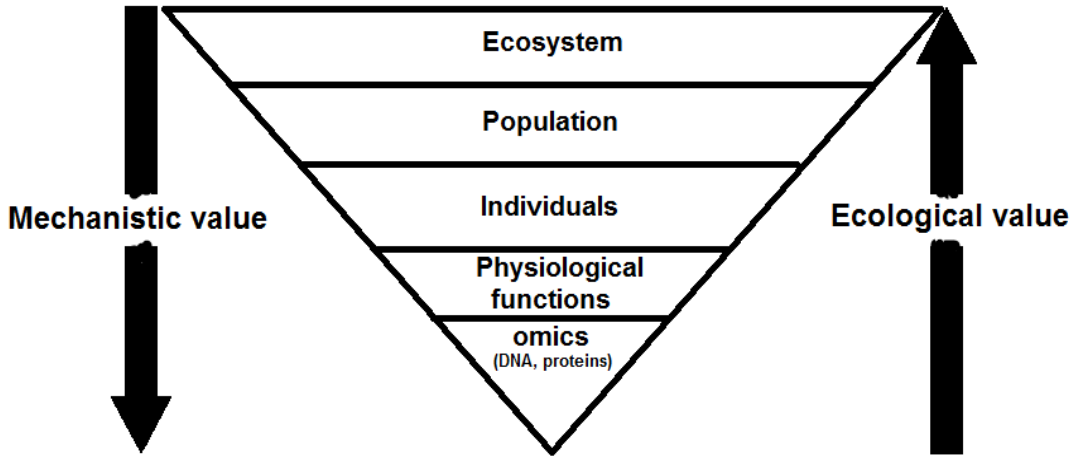


Figure 1.13. Representation of the effect that the focus of a study has on its level of mechanistic/ecological relevance. *This diagram represents a concept in ecotoxicology whereby the wider the shape becomes, the higher the level of ecosystem organisation at which the results can be extrapolated. Divisions/sections of the shape represent levels of ecological hierarchy, the width of the section indicates its comparative size and the direction of an arrow represents an increase in the information provided for a particular goal*. Redrawn and adapted from Snape *et al*., (2004).

Chapter 2: *Experimental Organisms; Husbandry and Biology*

The aim of this chapter is to describe 'general' methods used throughout the rest of the thesis, including basic husbandry of test species, associated considerations, and specific cell culture techniques.

The ways in which *B.glabrata* differs from some of the more commonly encountered model species used in ecotoxicology will be described in order to elucidate why, in certain cases, 'standard' approaches (i.e. to cell lines) will not work, due to the unique biology of molluscs.

2.1. *Biomphalaria* Husbandry

The majority of departments working with *B.glabrata* are only concerned with culturing snails as a means of producing cercariae to maintain the parasite life cycle for work on adult worms. In my research, snails were needed to support the growth and development of snail stages of *S.mansoni* and also to collect healthy hemocytes for *in vitro* assays.

Proper husbandry of snails is critical to ensure the reduction of confounding factors which may influence experiments. A range of environmental factors, described in the following sections, have previously been reported to affect the number and function of hemocytes taken from laboratory-reared molluscs (Lacoste *et al*., 2002; Gagnaire *et al*., 2006; Yu *et al*., 2009).

2.1.1. Diet

Wild *B.glabrata* snails feed on a large variety of items, the majority is vegetation but the snails will eat most organic material from which they can extract nutrition, including decaying animals, algae and mud. A large number of foods have been reportedly used in the laboratory. Although romaine lettuce is probably the most common, it is rarely used alone due to its poor nutritional value. Indeed, snails with a supplemented diet are known to grow as much as 4 times larger and lay 7 times as many eggs (Eveland and Haseeb, 2011; Tucker *et al*., 2013).

Among the reported supplemented diets are mud, algae, grass, shoots from wheat and barley, dog food, chicken food, powdered milk and thyroid/endocrine gland

extract (Eveland and Haseeb, 2011; Tucker *et al*., 2013). Many of these are relatively convoluted preparations, often using very precise ratios. For my studies I preferred to rely solely on commercial fish flakes (TetraMin®). Fish flakes have the advantage of being cheap, easy to use, extremely nutritious and edible to hatchlings (Tucker *et al*., 2013). The flakes had previously been tested in our laboratory for estrogenic activity and were free of know estrogenic chemicals which may affect physiological responses in animals.

One disadvantage of using fish flakes was their propensity to rapidly decay and pollute the water. For this reason, snails were kept in a flow-through water system to prevent water stagnation and were cleaned regularly. Feeding occurred 3 times a week. At each feeding event the quantity of food was limited to the amount that the snails could consume within a single day.

2.1.2. Photoperiod

Snails were maintained in a 12-hour light/dark cycle typical of most laboratories. Very little is known about the optimum photo period for *B.glabrata* in captivity, and therefore attempts to recreate its natural conditions are usually preferred.

2.1.2.1. Photoperiod for infected snails

Snails infected with *S.mansoni* are usually kept in constant darkness during the period of parasite development. This is achieved by placing a dark blanket over the tanks. This process is necessary to prevent the premature release of infective cercariae for health and experimental concerns, since light is used by the parasites as a cue to shed from the snails, something which is presumably an evolved mechanism to ensure maximum likelihood of infecting its diurnal final host. While snails will still shed some cercariae in the dark, the level is much reduced (Tucker *et al*., 2013).

There is little evidence to suggest that maintenance of infected snails in constant dark (or light) has any negative effects on the snail or parasite (Tucker *et al*., 2013). It has been reported that the snails require a minimum of 8-hours light for breeding.

However, infected snails are typically castrated by the parasite, so optimum breeding is difficult to determine for covered/infected snails (Webbe and James, 1971).

2.1.3. Pests

Aside from *S.mansoni, B.glabrata* is naturally subject to many other invertebrate pests including ostracods, oligochaetes, certain types of bacteria and even other trematode species (Eveland and Haseeb, 2011). In practice, it is impossible to ensure complete removal of unwanted invertebrates during routine maintenance of snails and attempting to do so could cause more harm than good, since many similarly sized organisms are symbiotes beneficial to snail survival.

Perhaps the most persistent pests in the laboratory are rotifers. Rotifers are not thought to affect snails directly, but do indicate poor general conditions (Eveland and Haseeb, 2011). Rotifers are of considerable concern when maintaining and studying infected snails. At least one species of rotifer is known to emit a chemical which can partially suppress cercarial release and even paralyze shed parasites (Lewis *et al*., 1986).

I was able to maintain a minimal level of biological contamination by regular cleaning and the use of flow-through tank systems. For the majority of work, rotifers (if they are present) do not appear to cause obvious problems. When raising snails for infection studies I took extra precautions, such as bathing egg masses (which would become the experimental snails) in 70% ethanol for 5 seconds followed by a distilled water rinse. This was repeated 3 times and does not appear to have any detrimental effects on subsequent snail development.

2.1.4. Water

According to the literature, the optimum water temperature for normal maintenance of *B.glabrata* is between 24-28°C (Stirewalt, 1954; Chernin and Schork, 1959; Sturrock and Sturrock, 1972).

Submersible aquarium heaters were found to be suitable for individual tanks, but for larger-scale maintenance it was easier to use a specially designated room that could

be kept at a precise temperature, or have circulated water supplied pre-heated, as in our laboratory.

Higher temperatures (up to 30°C) are sometimes used to accelerate the growth of snails and/or their parasites, but this often results in increased host mortality (Eveland and Haseeb, 2011; Tucker *et al*., 2013). Snails will survive for extended periods at temperatures as low as 20°C but growth is substantially reduced and parasitized snails will display decreased cercarial shedding and can even lose the infection entirely (Stirewalt, 1954; Tucker *et al*., 2013).

As with most metazoan aquatic organisms, snails rarely survive beyond a day in chlorinated tap water, but removal of chloramines usually makes this water acceptable (Eveland and Haseeb, 2011). Tap water is often left to 'age' by letting it stand for around a day, which is believed to allow harmful components, like chlorine, to evaporate off. Another method includes the addition of tap water conditioners which bind and neutralize toxic substances. For larger-scale maintenance and with flow-through systems like ours, it is best to have the water treated using a filtration system such as reverse osmosis (RO). RO water is reconstituted with minerals and salts to prevent the snails developing fragile shells. Small fish such as guppies are sometimes kept in the recirculation tank to help condition the water by encouraging the growth of beneficial bacteria from their waste and to eat some of the larger snail pests (Ulmer, 1970).

Precise pH levels are rarely considered when rearing snails and there is little information on optimum levels. However, it is known that *B.glabrata* can tolerate a wide pH range (4.9-8.9) without adverse effects (Chernin and Schork 1959; Webbe and James 1971; Eveland and Haseeb, 2011).

2.1.5. Density

As with most aquatic species, *B.glabrata* displays density dependent growth. A number of studies have attempted to determine the density for optimum growth and fecundity. Within the genus *Biomphalaria,* considerable differences have been found between species, with *B.straminea* apparently being able to live comfortably in twice the density of *B.glabrata* (Barbosa *et al*., 1992).

For uninfected *B.glabrata* with a shell diameter greater than 5mm it is typically reported that a density of no more than 10 snails per litre is optimal (Thomas and Benjamin, 1974; Tucker *et al*., 2013).

Regarding the optimal density for growth, Thomas and Benjamin (1974) have suggested that under certain circumstances a positive feedback effect of higher density can occur due to increased facilitation of metabolites, ions, food, increased tactile/visual stimulation between individuals and increased mating behaviour. In contrast, they also found that negative feedback can occur in static conditions when the density is higher than optimal, as a result of greater food competition and greater accumulation of toxic waste.

Regardless of the optimal density, practical considerations will always be a factor in the laboratory and it is sometimes necessary to make concessions due to space and/or safety. Tucker *et al.*, (2013) suggest that infected snails should be kept at higher densities as the presence of large volumes of potentially infected water could pose an unacceptable risk.

In our laboratory, stock snails are routinely kept at densities lower than 10 snails per litre. When performing an *in vivo* study optimal density is more precisely assessed according to the specific conditions and experimental requirements (section 7.3.1).

2.2. *Biomphalaria* cell culture

The importance of hemocytes as immune effectors in molluscs has led to a considerable body of literature regarding their *in vitro* culture and manipulation. These techniques have significantly contributed to my knowledge of their functions under various conditions (Boulo *et al.*, 1991; Yoshino *et al.*, 2013).

As with mammals, established cell lines exist for many insects. In contrast, only a single cell line for molluscs exists, it was derived from *B.glabrata* embryos and is known as ‘Bge’ (Hansen, 1976; Yoshino *et al*., 2013).

While Bge cells share some of the same characteristics as hemocytes (i.e. the ability to encapsulate sporocysts *in vitro*), they are not ontologically related (Yoshino and Coustau, 2011). Since Bge cells cannot be used as reliable substitutes for hemocytes,

and hemocytes cannot be cultured long-term, it was necessary to obtain primary hemocytes directly from the snail for my research.

Molluscan hemocytes are a notoriously difficult cell type to manipulate *in vitro*, therefore, a number of different methods have been developed to support the short-term survival and normal physiological function of hemocytes outside of the snail. *B.glabrata* hemocytes have a great propensity to clump to each other, often irreversibly, and also adhere readily to many types of substrate including collection vessels (Fryer and Adema 1993). Another aspect of hemocytes which hinders their manipulation is the fact that processes such as centrifugation often kill the cells or cause them to form a pellet from which they cannot be separated. This is in contrast to many mammalian cells lines which can be centrifuged at high speeds to form a pellet, but can still be readily separated and retain normal function afterwards (Fryer and Adema 1993). These factors present obstacles to the separation of hemocytes from hemolymph. This being said, a number of techniques have been specially developed for working with hemocytes and although the methods are often more time consuming and delicate, they do permit the use of hemocytes for *in vitro* experiments.

2.2.1. *Biomphalaria* cell buffers

By far the most widely used buffer for working with *B.glabrata* cells is Chernin's balanced salt solution ('CBSS'; Chernin, 1963). CBSS was first developed to support the *in vitro* culture of *B.glabrata* heart explants and was designed to mimic the salt composition, osmolarity and pH of the hemolymph (Table 2.1).

Hemocytes behave normally in CBSS and display phagocytosis and encapsulation even in the absence of hemolymph (Bayne, 1980). CBSS was the primary buffer used here for *in vitro* maintenance and manipulation of hemocytes and was also used as a carrier for chemicals or other materials which hemocytes were exposed to (Section 2.2.2.2).

Because hemocytes often cannot be separated after centrifugation, Fryer and Adema (1993) used buffers originally developed for another snail species (*lymnaea stagnalis*) to encourage *B.glabrata* hemocytes to reversibly detach from the culture

vessel, changing from a flattened shape with filopodial projections to a rounded cell. The buffers, which contain different chelating agents (α-CE; EDTA, α-CC; caffeine) that can maximize either detachment or survival, are able to round-up cells which have spread on glass and also to reduce clumping after low speed centrifugation. The buffers proved useful in my work and the reported reduction in phagocytosis caused by the buffers was of little concern, since in my studies the immune responses *in vitro* were completed before the addition of the buffer and hemocytes were not used after they had been detached other than to fix/analyse.

Table 2.1. Composition of buffers used in *B.glabrata* hemocyte *in vitro* culture and manipulation. *Of the three buffers described, CBSS is intended for normal hemocyte culture, while α-CE and α-CC were formulated for the purpose of manipulation*. Table adapted from Fryer and Adema (1993).

Chemical	**CBSS:** standard snail cell buffer (g/L)	**α-CE**: Haemocyte anti-clumping buffer (optimum cell retrieval) (g/L)	**α-CC:** Haemocyte anti-clumping buffer (preferential for phagocytosis studies) (g/L)
NaCl	2.8	1.7	1.7
KCL	0.15	-	-
Na_2HPO_4	0.7	-	-
$NaHCO^3$	0.05	-	-
$CaCl_2$	0.53	-	-
$MgSO_4$	0.45	-	-
Glucose	1	3.6	3.6
Trehalose	1	-	-
$Na_3C_6H5O_7$ (sodium citrate)	-	1.5	1.5
Citric acid	-	0.96	0.96
EDTA	-	0.58	-
Caffeine	-	-	4.85

2.2.2 Hemolymph collection

Many studies working with mollusc hemolymph do not consider sterility since it is essentially aseptic and long term culture is not possible as cells only survive for 2-3 days outside the animal. These factors combined reduce the likelihood of problematic bacterial infection (Yoshino *et al*., 2013). Nevertheless, it is still prudent

to collect sterile hemolymph where possible, in order to avoid any influence that bacteria may potentially exert, especially for immunological assays.

The following section describes a protocol, adapted from Fryer and Bayne (1995) which was used to obtain sterile hemolymph for my experiments.

Snails were collected and the shells dried with paper towel and then carefully swabbed using cotton buds soaked in 70% ethanol, taking particular care to clean inside the shell whorl. After air drying on paper towel for 1-2 minutes inside a laminar flow cabinet, snails were then placed in an autoclaved beaker containing sterile filtered water (maximum of 18 snails per 100ml) plus 2% antibiotic-antimycotic solution (10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 ptg/ml amphotericin B; Sigma-Aldrich, UK) and left for 45 minutes at 27ºC. After this time, snails were carefully removed from the beaker using sterile forceps and placed on a fresh paper towel inside the cabinet to dry for 1-2 minutes. The shells were again dried with paper towel, swabbed with 70% ethanol and the snail placed in an autoclaved glass petri dish. After the ethanol had evaporated (within 30 seconds) the hemolymph was extracted with a sterile scalpel according to the 'headfoot puncture protocol' described by Connors and Yoshino (1990). Hemolymph was allowed to collect for only a short period of time (< 5 minutes) to avoid cells adhering to the dish. Hemolyph was then extracted from the dish using a sterile p200 pipette and added to a falcon tube through a 70µm cell strainer (Falcon® Cell Strainer; Fisher Scientific Co., UK) to prevent shell debris or cell clumps passing into the sample. Since the collection process can take a reasonable amount of time (depending on the number of snails being sampled) the tubes were held on ice to prevent clumping prior to the assay.

This process enabled the collection of hemolymph that was free of visible contamination and debris. Debris is commonly found in hemolymph samples (e.g. shell fragments, clothing fibres, pollen) and these compromise studies since hemocytes will naturally attempt to encapsulate such foreign objects (Figure 2.1). An alternative reported method is to pool the hemolymph and allow it to rest for 5-10 minutes. The heavier debris will settle to the bottom while cells can be extracted from the upper column/supernatant. However, this method results in considerable aggregation between cells, requires very delicate pipetting to avoid collecting the

settled debris and reduces the final cell yield. Therefore, passage through a 70µm filter was used since individual hemocytes will pass through while larger objects (which will not be hemocytes or will be clumped cells) are retained.

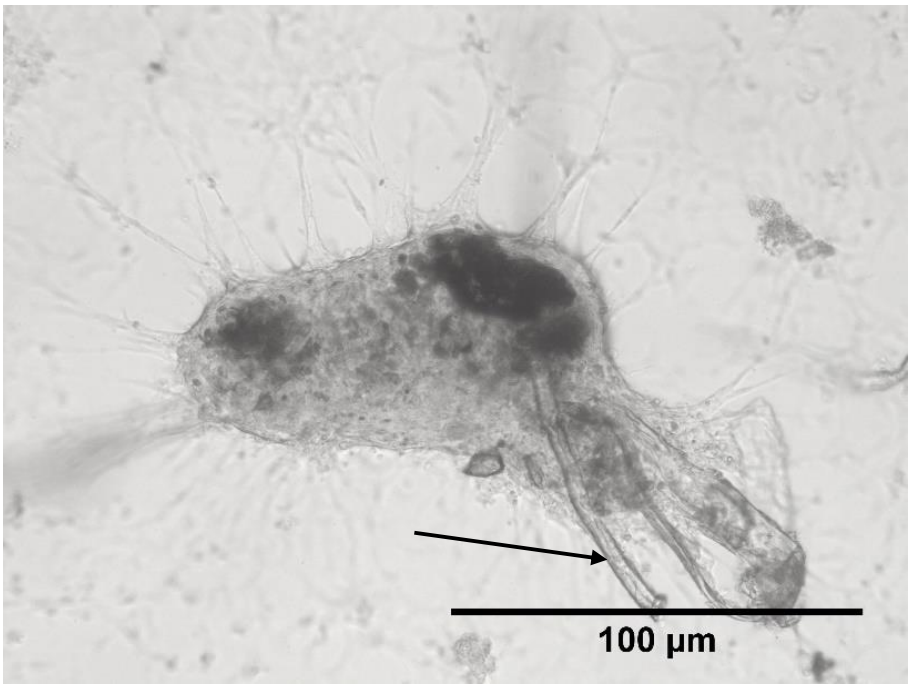


Figure 2.1. Photograph of *B.glabrata* hemocytes attacking and encapsulating a piece of foreign debris. *This image demonstrates the propensity of these hemocytes to clump around objects of foreign nature or even shell fragments*. Image taken at 400x, arrow indicates the debris.

2.2.2.1. Pooling

Variation in the response of snail hemocytes from different individuals and even between different cells from the same individual, is relatively high compared to other types of cells (Cuenot, 1914; Ashton-Alcox and Ford, 1998; Ford and Paillard, 2007). In *B.glabrata,* responses between individual snails can sometimes show considerable, and often unexplainable, variation. For example, the majority of cells from one animal may spread readily on contact with glass while the majority of cells from another remain rounded (Connors and Yoshino, 2012). Such variation introduces problems when undertaking immunocompetence assays (Oehlmann and Schulte-Oehlmann 2002).

One of the most commonly reported solutions to this problem is through the technique of pooling. Pooling involves mixing hemolymph samples from several

animals and splitting the sample into sub-samples on which tests are carried out (Figure 2.2). This method is used as it removes the inter-individual variation between snails and is reported in a large number of studies (Amen *et al*., 1992; Bakry *et al*., 2012b).

Pooling of hemocytes from a number of individuals has the potential to reduce biological variability by measuring the average of a population instead of the response of each individual. This is why it is an appealing option for those working with highly variable animals (Lee, 2008). However, the problem that often arises with this approach is one of pseudoreplication, and to understand this we must consider what our experimental units are i.e. the units upon which statistical analysis is based.

A typical definition of the experimental unit is "the smallest division of the experimental material that could, in principle, receive any of the treatments used in the study" (Cox and Reid, 2000; Langton, 2012). Many researchers suggest that by pooling from several animals the pool itself becomes the experimental unit rather than the individual snails and that sub-samples of the pool do not constitute true biological/statistical replication but rather technical replication. This is because the cells come from the same population (the pool) and are subject to similar conditions. The aim of statistical analysis is to make conclusions based on a population, this requires a representative sample of individuals (snails) from a population to be taken. The effect of pooling is to average out the biological variance which we are interested in and this leads to false estimations of significance. However, this kind of sub-sampling is still useful in that it helps improve the precision of each measurement by reducing the effect of sampling error or noise (Langton, 2012).

By this definition, any work based on a cell line could be considered pseudoreplication since all the cells originally came from the same animal and are therefore related. However, this is often not considered for a number of reasons, not least practicality.

When a group of cells from individual animals are pooled (as is often the case in molluscan hemocyte work), one could argue that each cell is, in theory, capable of being randomly assigned to any one treatment. Therefore, it is the cell which constitutes the experimental unit and not the pool.

Another approach is to treat the experimental unit as the smallest unit upon which a measurement can be made, rather than the smallest unit to which a treatment can be applied (Robinson *et al*., 2006). In theory, this definition removes the issue of pseudoreplication if using an assay capable of performing single-cell analysis, since the measurable 'population' could now include all the cells in the pool. Single-cell measurements which can be made by instruments (such as imaging flow cytometers or comet assays) can help to reveal information which is potentially obscured when averaging a population of cells (Gordon *et al*., 2007; Shanbhag and Rao, 2003).

In reality, there is no fixed definition of the experimental unit. Some authors have stated that pseudoreplication is a 'biological issue' and that what constitutes a true replicate depends more on the biology of the species of interest and the questions that are being asked rather than an entirely statistical approach (Ruxton and Colegrave, 2011). In some circumstances pooling is unavoidable. For example, individual cell yields may be too low to study and this is often the case with smaller snails.

One compromise that is commonly used in hemocyte immunocompetance assays, (and an approach that I have typically used), is to make separate pools of hemocytes (Fryer and Adema, 1993). For example, three independent pools of hemocytes could be prepared, each consisting of cells from different animals (Figure 2.2). This approach helps to provide enough hemolymph, reduces the effects of unresponsive snails and provides sufficient biological replicates by most definitions (Kendziorski *et al*., 2005). In this example the experimental unit would be each separate pool (n =3; Figure 2.2).

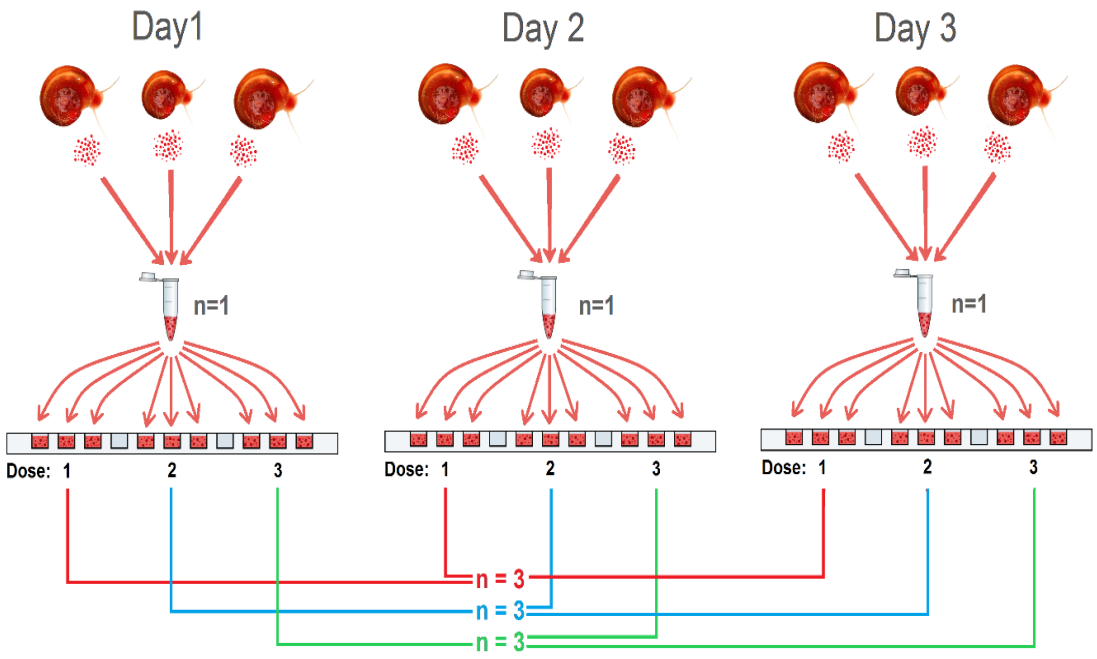


Figure 2.2. Diagrammatic example of the process by which my technical and experimental units were generated. *Hemolymph from individual snails was pooled, mixed and then allocated to a specific treatment to form a single experimental unit. This was repeated on a different day, with different animals, to produce independent replication in the experimental unit. The three snails and converging arrows indicate the pooling of hemocytes from each snail into a tube, the following nine arrows indicate how samples from the pool were allocated to wells (with dose 1-3 representing different levels of a chemical treatment to be received by the above three wells), lines of the same colour (red, blue or green) show how wells contribute to three experimental units for each dose (n=3).*

One final aspect of the experimental design that is useful when applying these types of assay is to test the actual variation associated with pooling by calculating both the inter and intra-individual variation of test animals (Hegaret, 2003).

2.2.2.2. Incubation with test chemicals

Adding test chemicals directly to hemolymph may cause significant reductions in cell viability since these chemicals are typically purchased dissolved in some form of volatile solvent (e.g. ethanol). Even if the final percentage of solvent present in a sample of hemolymph were the same, it is likely to induce greater toxicity if added directly to hemolymph rather than diluted in a physiological buffer.

In my *in vitro* experiments, serial dilutions of test chemicals (discussed in Chapter 3, section 3.2) were made in CBSS from concentrated solvent stocks. In order to avoid

excessive manipulation of hemocytes, and the problems this causes (Section 2.2.1), the desired concentration of test chemical in the hemocyte sample was achieved by making a double strength (2x) stock in CBSS and mixing this with an equal volume of hemolymph. This resulted in a solution of 50% hemolymph and 50% CBSS/test chemical. Many of the original *in vitro* experiments concerning *B.glabrata* hemocytes maintained the cells in 100% CBSS with no observable detrimental effects (Section 2.2.1). The effect of the residual solvents was studied and is reported in Chapter 3, section 3.4.

2.3. Parasite culture

Several of my assays involved working with the snail-related stages of *S.mansoni* and it was therefore necessary to be able to harvest and culture the parasites. *In vitro* interaction assays between hemocytes and intra-molluscan stages of the parasite required the harvesting and hatching of eggs and the subsequent collection and transformation of miracidia as they would exist initially inside the snail.

2.3.1. Safety considerations

Only the cercarial stage of *S.mansoni* is infective to humans and it is designated as a category 2 human pathogen hazard according to the Health and Safety Executive Advisory Committee on Dangerous Pathogens. It was deemed good practice to treat any infected snails as potentially hazardous, especially since cercarial shedding time differs between individual snails. Category 2 pathogens are defined as having the potential to cause human disease but are unlikely to spread, and for which there currently exists effective prophylaxis (HSE, 2013).

My work with infected snails/cercariae was carried out in a licensed BSL-2 laboratory at the London School of Hygiene and Tropical medicine. Latex gloves were worn at all times when in the BSL-2 laboratory, as were long-sleeved coats since cercariae could potentially land on exposed skin if water were accidentally splashed. If infectious water is thought to have contacted skin then the affected area would be sprayed immediately with 70% ethanol to prevent an infection, since the parasite takes several minutes to penetrate skin (Tucker *et al.*, 2013).

Handling of infected snails should be kept to a minimum and bare hands never used. Snails were removed with a sieve if cleaning tanks and the waste-water was disinfected with bleach before disposal. All material in contact with snails was washed in 70% ethanol, avoiding the use of toxic chemicals where the implements were to be re-used.

While work with the miracidial and sporocyst stages does not provide a direct hazard, and is not regulated, there does exist an extremely small risk. If miracidia were to somehow find their way into the aquaria of uninfected snails, a snail could then become infected and potentially produce thousands of cercariae with the potential to infect people without their knowledge. While the chain of events required for this to happen are extremely unlikely to occur, work with miracidia did not occur in any room containing uninfected snails and clothing was changed after working with miracidia, especially if proceeding to work near uninfected snails.

The first potential symptom of an infection would occur shortly after cercarial penetration of the skin, with very mild cutaneous lesions appearing at the site, but this typically will go unnoticed (Tucker *et al*., 2013). 2-3 weeks' post-infection, a febrile response to the presence of larvae in the lungs may occur and a more acute response (known as Katayama fever) may manifest with the laying of the first eggs after around 5 weeks (Tucker *et al*., 2013). Chronic Schistosomiasis takes several years to develop and can ultimately lead to liver failure and portal fibrosis (Tucker *et al*., 2013). Despite these risks, *S.mansoni* is easily diagnosed by the presence of eggs in faeces and can be very effectively treated with a single dose of praziquantel (Tucker *et al*., 2013). Praziquantal may also be taken prophylactically by someone who suspects that they may have been exposed to cercariae but before the first eggs are laid.

2.3.2. Mouse stage and egg collection

In the laboratory, mice (sometimes also rats or guinea pigs) are used as the final mammalian host in order to maintain the life cycle of the parasite as they support the development of adult worms and eggs in much the same manner as would an infected human.

To obtain parasites for my work the infection of mice was carried out under the United Kingdom Animal's Scientific Procedures Act 1986, with approval from the London School of Hygiene and Tropical Medicine Ethics committee.

Mice (purchased from Charles River, Margate, Kent, UK) were typically infected by subcutaneous injection of approximately 100 cercariae. The infection was allowed to mature for 7-8 weeks, at which point the animals were euthanized with intra-peritoneal injections of Tiletamine/Zolazepam (800 mg/kg) and Xylazine (100 mg/kg). Euthanasia may be brought forward if the animals are visibly suffering from the infection.

Once the mice have been euthanized, the adult worms are collected by perfusion of the hepatic portal system and the eggs collected by removal of the liver.

2.3.3. Egg extraction and hatching

The mouse liver containing the parasite eggs can be stored at 4^{o}C for 48 to 72 hours but recovery of viable miracidia will reduce steadily over time. Eggs were typically harvested on the same day that the liver was removed to ensure optimum yields.

The liver was first washed with a solution of 1.2% NaCl to remove excess tissue and then placed on a metal sieve with a 180 µm pore mesh. The sieve was placed on top of a conical measure flask. The tissue was manually homogenized using a porcelain pestle, with the addition of more NaCl solution to aide passage through the mesh. Once all the tissue had passed through the sieve the flask was topped up with 1.2% NaCl to a final volume of 200ml, at which point it was placed in a fridge at 4^{o}C for 30 minutes. The combination of saline solution and low temperature prevents premature hatching of the eggs. After this settling period a tissue sediment was formed, the flask was removed and half of the supernatant was carefully poured off and discarded, ensuring minimal disturbance of the sediment. The flask was then topped up to 200ml with fresh 1.2% NaCl and returned to 4^{o}C for a further 30 minutes. This process was repeated 2-3 times until the supernatant appeared clear at which point the majority of the solution was poured away (leaving the sediment undisturbed) and replaced with freshwater.

The contents of the flask were then poured into a large petri dish in a room with an ambient temperature of 27°C and a lamp placed directly above. After 30 minutes the miracidia will start to hatch with peak rates occurring after around 45 minutes. Miracidia were then collected for subsequent snail infection.

2.3.4. Miracidia collection

For routine collection of miracidia (i.e. for use in snail infection) the parasites were taken directly out of the hatching dish using a pipette. For *in vitro* work it was necessary to prepare a much cleaner sample. The homogenization process often yielded many small fragments of mouse liver and other organic particles. These particles were often smaller than the eggs and so could not be filtered out with the sieve. Such fragments do not influence snail infection but pose serious problems to other work. *In vitro* interaction assays between hemocytes and parasites will be disrupted by such tissue as the hemocytes will instead attack the foreign mouse tissue, leading to reduced numbers available for parasite encapsulation (Figure 2.1). Any attempt to perform molecular work or protein quantification would also be severely compromised by the presence of mouse tissue.

For these reasons an alternative protocol for miracidia collection was developed. The method was an adaptation of that described by Jurberg *et al*., (2008). Their original method was intended as a means of performing more accurate diagnosis of *S.mansoni* infection based on human faecal samples, but they also alluded to its potential as a way of obtaining contaminant-free miracidia samples for immuno-histochemistry, molecular, and biochemical studies.

The new approach made use of the positive geo and phototaxic responses of miracidia, so the parasites were, in essence, separating *themselves* from any debris (Figure 2.3).

2.3.4.1. Lighting

In order to achieve a point-source of light a wooden box to house the hatching device was constructed. This box had a small hole in one side which exposed only the

collection jar to light, thereby creating a preferential location for the parasites to move toward (Figure 2.3B). The inside of the box was painted black and a foam seal was attached around the hole to reduce the chances of light entering the main flask. While room lighting works sufficiently, I also used a swing-arm lamp to direct light to the collection jar from the side, so as not to shine light through the hole and into the box. A small amount of light inevitably entered the main flask as the collection jar was transparent. However, this was actually advantageous as it formed a phototaxic gradient without which the parasites might be unable to locate the main light source.

2.3.4.2. Construction of the flask

A 500ml glass side-arm flask was first adapted using non-toxic silicone aquarium glue to attach a p20 pipette tip to the side-arm exit (Figure 2.3A).

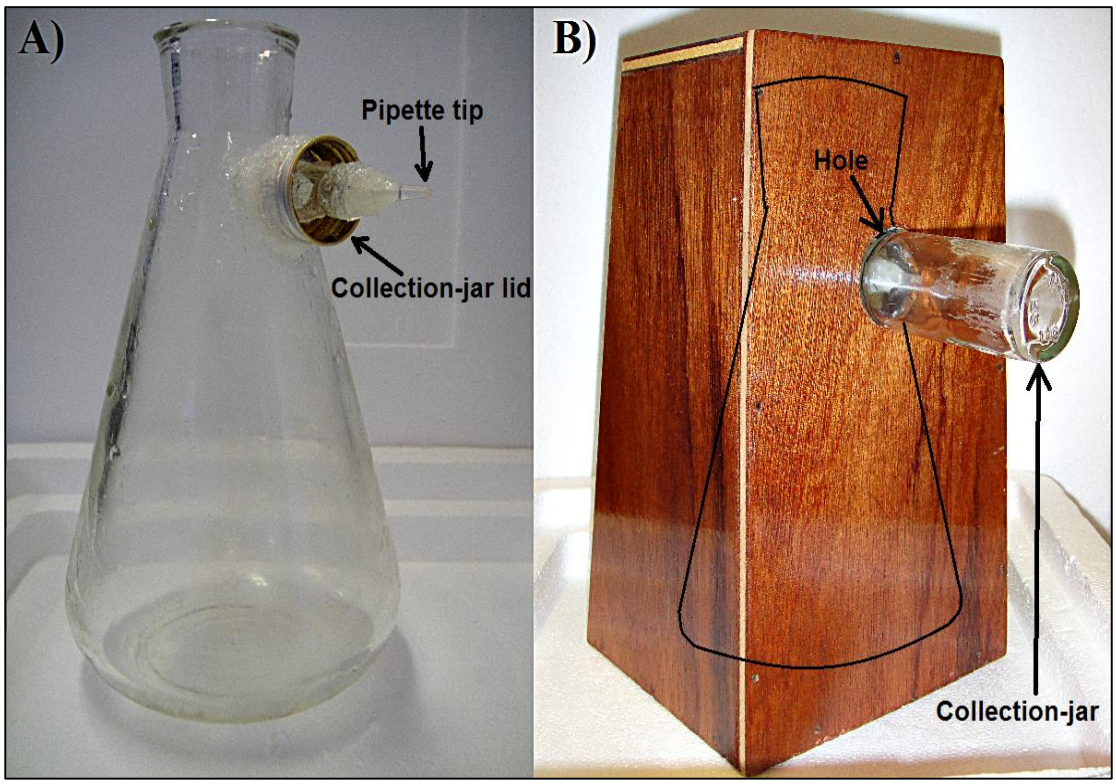


Figure 2.3. Photographs of my miracidia collection device. ***A)*** *500ml side-arm flask for collection of liver homogenate. The side-arm was adapted with the addition of a bottle cap and p20 pipette tip* ***B)*** *Wooden box constructed to house flask, the collection bottle can be seen attached and extruding so that light is only exposed to the collection jar where miracidia will pool themselves*.

The use of a p20 tip serves as a ‘funnel-trap’ whereby miracidia can easily pass thought the wide end into the bottle, but are unlikely to be able to return into the main flask through the narrow end.

A hole was drilled through the lid of the collection jar and the side-arm passed through (Figure 2.3A). The glass jar was then able to be attached and removed with ease.

2.3.4.3. Chemical gradient

In order to further encourage the movement of miracidia, transformation media was added to the collection jar. The transformation media (Section 2.3.5) encourages miracidia to immediately cease movement and begin transformation into sporocysts, thus further reducing the likelihood of them escaping. If transformation of miracidia was not required (i.e. when wishing to infect snails) then the collection jar could be filled with normal fresh water, as with the main flask. In order to encourage a chemical gradient between the two chambers, the flask was placed at an angle of 15^{o} and thus, coupled with the position of the side-arm, providing a geotaxic gradient for miracidia to follow.

Basic experiments were performed to test the extent and stability of the gradient by placing red food colouring in the collection jar and leaving it for 1 hour to determine how fluid traveled between the two chambers. Visual inspection showed a very fine ‘trail’ of liquid passing from the collection jar into the main flask (Figure 2.4). This appeared to be ideal as it would act as a chemotactic trail encouraging the miracidia toward the desired destination while not introducing sufficient media into the main flask as to encourage transformation before the parasites reached the collection area.

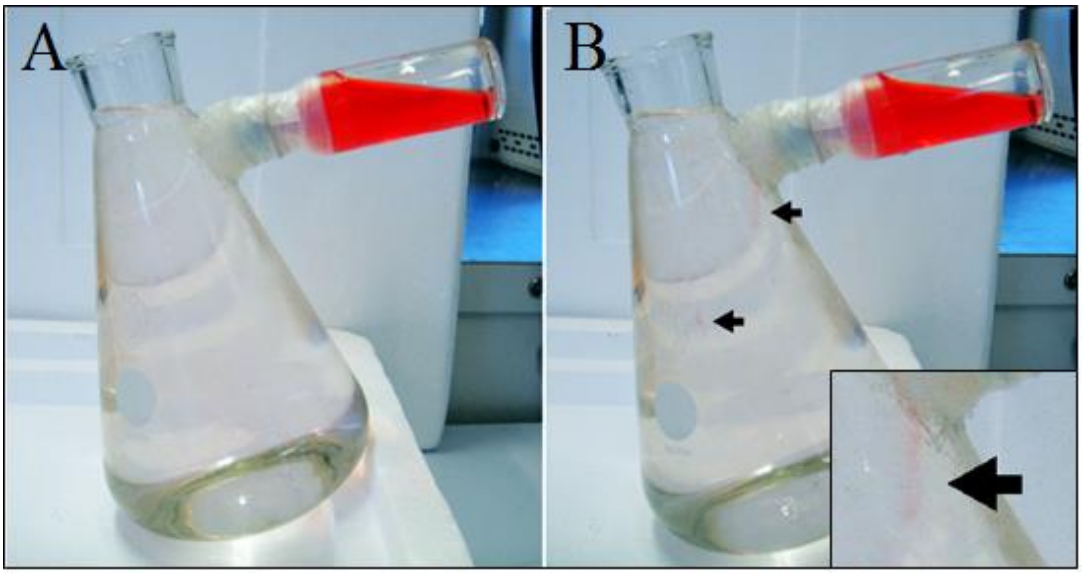


Figure 2.4. Demonstration of the chemical gradient formed between the miracidia collection chamber and the main flask. *Red dye was used to allow observation of the gradient,* ***A)*** *after 0 hours* ***B)*** *after 1 hour. Arrows indicate the dye trail – the enlarged section shows the trail in more detail.*

2.3.4.4. Pressure gradient

Achieving the correct pressure gradient between the two chambers was initially problematic. Attaching the chambers without adjustment meant that miracidia were unable to pass through the pipette tip as a result of high pressure in the collection jar and also the possibility of air bubbles blocking the tip exit. To amend this, I filled the main flask with water, then I attached the collection jar, which contained the media, and finally applied a bung to the top of the flask. A pre-compressed 10ml plastic syringe was passed through a hole in the bung and very gently the syringe was drawn up until I observed media passing into the main flask. This resulted in equalization of pressure between the chambers as shown by the movement of the red dye (Figure 2.4) and subsequent entry of the miracidia into the chamber.

2.3.4.5. Addition of sample

After the egg collection (Section 2.3.3), the egg/liver homogenate solution was poured into the main flask. Fresh water was added until the water level in the main flask was higher than the location of the side-arm. Angling the flask prevented any debris entering the collection chamber and helps establish the chemotaxis gradient

(Section 2.3.4.3). The solution was left to settle for 30 minutes, after which the collection jar was exposed to light.

2.3.4.6. Miracidia harvesting and transformation

Given the need to collect miracidia for subsequent *in vitro* transformation, an added advantage of this system was the use of transformation media in the collection chamber which immobilized the miracidia and enabled maximum yield. In an ordinary system (i.e. for snail infection) miracidia are collected before they lose their energy reserves and ability to infect. Since the collection vessel contained transformation media I was able to leave it for extended periods of time as any miracidia that entered would immediately begin to transform without requiring intervention. This meant that slower hatching parasites also had the opportunity to enter the collection chamber, therefore minimal numbers were lost.

The collection jar was very carefully unscrewed from the side-arm once miracidia were no longer observed entering and a second unmodified lid attached. The contents were then transferred to well-plates and left to complete transformation into sporocysts.

2.3.4.7. Findings

Quantitative analysis of the numbers of parasites collected using this method compared to the basic method of miracidia collection was not undertaken. Miracidia numbers were always adequate for my purposes using this method and crucially, the amount of liver contamination was extremely low. Miracidia responded very well and after 30 minutes could be seen in large numbers streaming through the pipette tip and into the collection jar. Miracidia entering the collection jar became immobile almost instantly, and subsequently transformed into viable sporocysts.

After miracidia had been collected they were used immediately to infect snails (for the maintenance of the life cycle or *in vivo* studies) or were collected in transformation media for subsequent use in *in vitro* assays.

2.3.5. Miracidia transformation media

It is possible for the snail immune system to attack any intra-molluscan stage of the parasite, even in susceptible hosts, however the primary response is usually directed toward the early sporocyst (Delgado *et al*., 2001; Goodall *et al*., 2004; Loker, 2010). In order to achieve a more realistic *in vitro* representation of the snail immune response it was necessary to use sporocysts and this necessitated the *in vitro* transformation of collected miracidia.

Sporocysts are defined as miracidia that have shed their ciliated plates and exhibit muscular squirming (Mann *et al*., 2010).

Sporocysts can be maintained *in vitro* for at least 14 days under basic conditions and potentially continuously, up to and including the production of cercariae, although this is considerably more difficult (Mann *et al*., 2010). For my purposes I was interested in early sporocysts around 6-hours after first contact with the medium as the ciliated plates have mostly shed and the syncytial tegument is formed (Figure 2.5). At this time the parasite is most vulnerable to elimination by the snail immune system (Pan, 1996; Peterson *et al*., 2009).

A number of different transformation media are reported in the literature but the most commonly used is simply the same CBSS used for hemocyte culture (Azzi *et al*., 2009; Peterson *et al*., 2009; Abou-El-Naga *et al*., 2014). I was unable to achieve sufficient numbers of miracidia to transform into sporocysts using CBSS. Despite multiple tests using separate batches of CBSS, carried out at two different optimum pH values reported in the literature (7.2 and 7.4), it was not possible to achieve sufficient transformation, let alone > 90% rates reported for CBSS (Walker and Rollinson, 2008; Azzi *et al*, 2009). Since CBSS worked very well for supporting hemocyte culture and untransformed miracidia, it is likely that this was due to differences in the parasite strain used, rather than the composition of the media.

Adult worms are routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum and 2mM L-glutamine (Mann, *et al*., 2010). I found that miracidia readily transformed at rates close to 100% in DMEM (Figure 2.5). Moreover, removal of the bovine serum did not alter the effectiveness of DMEM, which further simplified the method and reduced the cost. Other media have been reported as transforming miracidia with

similar success, such as MEMSE-J (which is an amino-acid composition similar to DMEM) and also RMPI-1640 (which is widely used in mammalian cell culture) (Kawanaka *et al*., 1983; Cook *et al*., 2009; Mann *et al*., 2010).

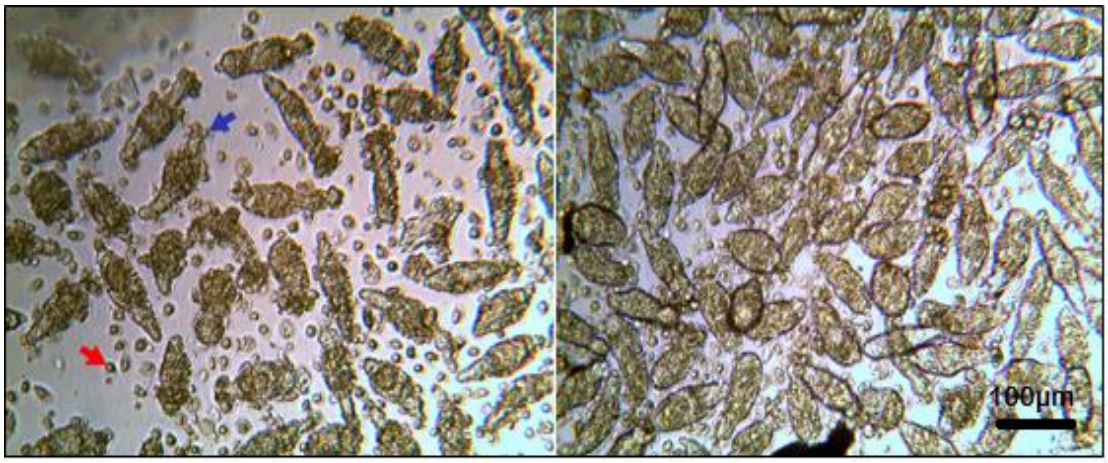


Figure 2.5. Miracidia developing into mother sporocysts in DMEM media. ***A)*** *10 hours after miracidia exposure* ***B)*** *24 hour after miracidia exposures. Blue arrow indicates ciliated plate still attached and red arrow indicates a fully shed plate.*

2.3.6. Infecting snails; background

For routine maintenance of the parasite life-cycle it is common to perform mass infections of snails. Mass infections involve pooling snails together in one beaker, followed by the addition of a large number of miracidia. The precise number of miracidia used depends on the desired number of infections per snail. This method has the advantage of being much less time consuming and is particularly useful for large scale infection. However, it is unsuitable for studies where precise control of the infection process is required. Mass infection cannot guarantee that each snail has equal opportunity to be infected by a specific number of miracidia. Indeed, some snails may not be infected while others may obtain heavy infections resulting in high mortality (Ismail *et al*., 2014).

For the *in vivo* work, a precise method of infection was used to ensure that each snail had the potential to be infected by the same number of miracidia. However, it is unlikely that this actually occurrs in practice, due to uncontrollable genetic differences in compatibility between individual snails and parasites.

2.3.6.1. Number of miracidia

Several studies have attempted to estimate both the optimum age at which to infect snails and the optimum number of miracidia to use in order to achieve high infection rates with low mortality. Makanga (1981) found that miracidial doses of 0, 1, 2 and 4 resulted in mortality rates of 5, 10, 40 and 75% respectively. Blair and Webster (2007) failed to find a dose-dependent relationship between miracidia number and snail mortality, but reported that mortality increased in exposed snails even if they did not develop an infection. It has been suggested that combating infection places a physical cost on the snail (Blair and Webster, 2007; Humphries, 2010). Grassi *et al*., (2001) found that the percentage of *B.glabrata* which developed an infection was consistent regardless of miracidia dose. Although there appears to be no definitive consensus regarding optimum number of miracidia, when individual snails are valuable (e.g. as experiments units or rare field isolates) it is generally considered good practice to use as few miracidia as possible.

For my experiments I chose to use 7 miracidia per snail in order to strike a balance between optimizing snail survival, while ensuring adequate infection rates. This number was approximately in the mid-range of those typically reported in the literature for experiments involving *S.mansoni* miracidial infections

2.3.6.2. Age of snails

In general, the age of the snail is correlated with shell diameter and therefore age and shell diameter are often used interchangeably to describe a snail (Borges *et al*., 2006).

As with miracidial dose, there is considerable discrepancy between reports of the optimum size of snails for infection, but unlike miracidial dose it is widely, if not universally, accepted that a correlation exists between snail size/age and mortality rates upon infection (Eveland and Haseeb, 2011). Infection with *S.mansoni* causes higher mortality in *B.glabrata* than in uninfected snails, regardless of age, but it has been consistently found that very young and very old snails are more likely to die after infection than differently-aged individuals.

Meier and Meier-Brook (1981) reported a mortality rate of 77% in 1-week old *B.glabrata* snails upon *S.mansoni* infection. This percentage decreased with increasing snail age but so did the infection success rate. Sturrock and Sturrock (1970) reported a higher mortality rate in 2-week old infected snails, which then decreased with age until 44 weeks, at which point mortality returned to a level similar to that of the 2-week old snails. In general, infected snails display increased mortality rates compared to uninfected snails and these are highest in snails of ages where mortality rates would naturally be expected to be higher regardless of infection. That is, infection exacerbates the mortality-associated risks of age (Humphries, 2010).

For routine maintenance of the life-cycle snails of approximately 10mm in diameter were typically selected (which equates to about 4-6 weeks of age post-hatch) to ensure sufficient numbers of infected snails while preventing excessive mortality. In experimental studies I did not discriminate based on size, and infected young adults of a similar age regardless of any discrepancies in size.

2.3.6.3. Snail infection protocol

Some miracidia may sustain damage during the hatching process and these parasites will settle to the bottom shortly after hatching (Eveland and Haseeb 2011). Miracidia should not be collected after 3-hours post-hatching as their glycogen stores and ability to successfully infect will begin to decline (Eveland and Haseeb, 2011). For snail infections, miracidia were collected between 45 minutes and 2-hours after hatching to allow sub-optimal parasites to settle to the bottom, maximum hatch rates to occur and glycogen stores to remain sufficient.

Prior to infection, snail shells were individually cleaned using a paper towel to remove any undesired microorganisms and excess mucus which can trap miracidia. Snails were placed individually into wells of a 6-well plate. Miracidia were placed into a large glass petri dish in water approximately 5mm deep and collected individually using a Gilson p20 pipette (Figure 2.6).

The method used to catch miracidia proved to be very effective in terms of collecting precise numbers. The pipette was set to a volume of 100µl and the plunger

depressed, the tip was then placed into the water containing the miracidia. Parasites were observed using a dissecting microscope and as individuals passed by the tip of the pipette the plunger was released, which drew in individual miracidia with minimal water. Another advantage of this method was that numerous miracidia could be collected in one go without having to expel the sample. This was achieved by allowing air bubbles to form inside the tip between each miracidia that was caught. The air bubbles prevent the previous parasite from escaping when attempting to catch the next (Figure 2.6).

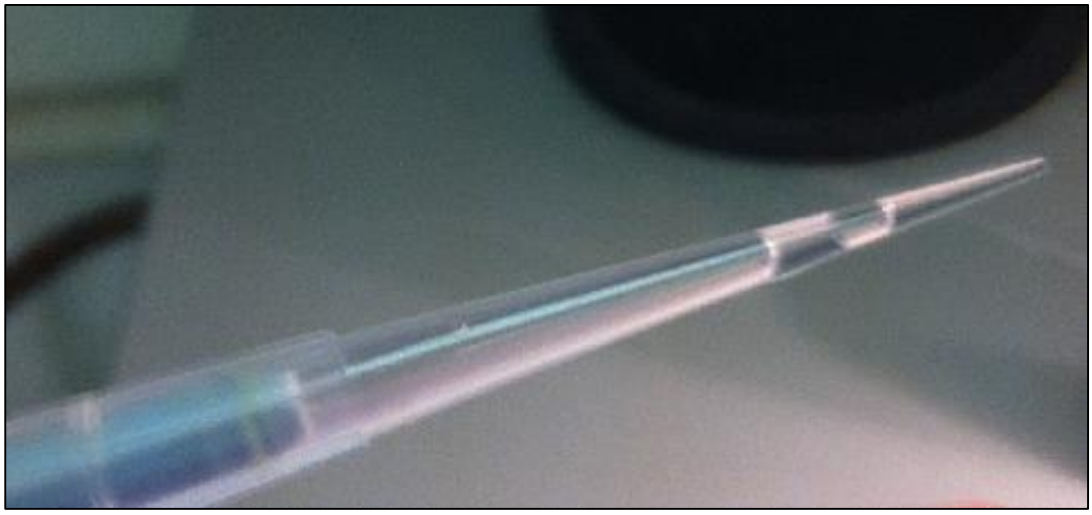


Figure 2.6. Photograph of pipette tip demonstrating my method for catching precise numbers of miracidia. *An air bubble, drawn in between each capture of a parasite, separates two columns of water, each containing a single parasite.*

In total, 7 miracidia were added to each well of the 6-well plate and freshwater was added until the well was full and the snail completely submerged. The plastic lid was applied and the plates left overnight at 27ºC to ensure maximum opportunity for infection. The following day, snails were removed from the well plates and placed into larger communal tanks. These tanks were draped in a black cloth during the course of the infection to reduce premature shedding. Snails were fed normally and the tanks inspected daily to remove any dead.

2.3.6.4. Shedding

The time at which the first cercariae are shed can be quite variable between individual snails depending on numerous factors. Lemos and Andrade, (2001) found

that *B.glabrata* exposed to 15 miracidia first began to shed on the 37th day post-infection, with peak shedding occurring between the 67th and 82nd day, after which the rates declined and shedding ceased at day 142 post-infection.

Temperature is perhaps the most important factor in determining parasite development speed and hence the date of the first cercarial release. Foster (1964) studied the effect of temperature on the time of fist cercarial shedding in *Biomphalaria pfeifferi* infected with *S.mansoni* and found a positive linear relationship between increasing temperature and time to first shedding (Table 2.2). While shedding was achieved earliest at the highest temperature (31.75°C), this came at the cost of increased mortality. Therefore, snails were maintained at 27°C, since this is closer to the natural mean daytime temperature at which the interaction between parasite and snail would naturally take place.

Table 2.2. Relationship between snail water temperature and mean number of days to first cercarial shedding. '*Temperature' refers to that of the water in which the infected snails are being kept and 'mean time to first shed' refers to the mean number of days until the first cercariae can be seen to emerge from the snail under microscopic investigation*. Table recreated and adapted from Foster, (1964).

Temperature (°C)	Mean time to first shed (days)
18	57
21	37
22.85	34
24.01	32
26.26	24.5
28.07	21.5
30.04	20
31.75	17.5

Snails can be observed for shedding daily, although there is little point in doing so before the third week post-infection. Alternatively, a specific day can be chosen based on knowledge of shedding dynamics, from which to plan a single large-scale shedding event. Assuming the snails are kept in the dark, then performing a large-scale shedding is a quick way to achieve a reasonable estimation of infection rates while reducing the frequency with which infected snails are handled.

For my work, snails were prepared for shedding in a similar manner to when infecting with miracidia (2.3.6.3). Shells were cleaned, including removal of excess mucus to avoid cercariae becoming trapped and individual snails placed in wells of a 6-well plate. The wells were filled with an equal volume of fresh water and the lids replaced. The plates were put under a lamp for 45 minutes, ensuring an equal distribution of light intensity between wells when quantifying differences between individual snails. After 45 minutes the cercariae start to emerge en-masse and at this point I removed all the water in the well and transferred it to a 50ml Falcon tube (Fisher Scientific Co., UK). The snail was rinsed with the same water to ensure no cercariae remained attached to the shell. The 50ml tube was mixed gently and six 5µl samples were taken with a p20 pipette then dotted onto a petri dish (Figure 2.7). This volume was chosen as a representative sample since cercarial numbers from a single shedding event can be in the tens of thousands and could not reasonably all be counted. To each 5µl sample, an equal volume of iodine solution was added (4g potassium iodide and 2g iodine in 100 ml distilled water). The iodine serves two purposes; it stains the cercariae dark yellow-brown so they are easily visible in contrast to the dish, and secondly it fixes them to the dish, immobilising and killing them (Figure 2.7).

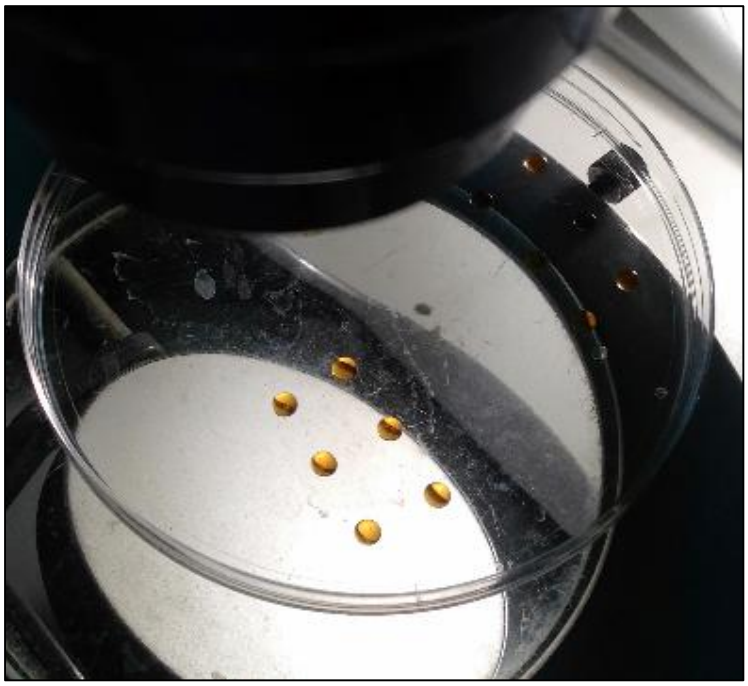


Figure 2.7. Photograph of my method for cercariae quantification**.** *Each yellow-brown dot is a 5µl sample of the water in which a snail was allowed to shed, mixed with an equal volume of iodine solution to immobilize the parasite and enhance contrast.*

The number of cercariae in each 5µl dot could then be counted and the process was repeated 3 times for each sample/snail. The number of cercariae in each dot was averaged and extrapolated for the total volume.

2.4. Alternative model: *Marisa cornuarietis*

In addition to *B.glabrata,* I also performed some basic studies using the hemocytes of another gastropod mollusc; *Marisa cornuarietis*. In recent years this species has gained attention due to its potential as a model organism for studying the effects of possible endocrine disruption in molluscs (Schulte-Oehlmann *et al*., 1995; Lyssimachou *et al*., 2009). Other elements of this specie's biology, such as its short generation time, transparent eggs, and large size make it a good candidate for toxicology and immunotoxicological studies (Sawasdee and Kohler, 2010).

2.4.1. Aim

The work conducted with *Marisa* was not intended to answer the overall thesis hypothesis, despite several overlapping aspects. Indeed, this particular work was borne out of the desire to apply the *in vitro* assays developed for *B.glabrata* to another species of mollusc in order to determine what, if any, differences in responses might occur between species.

The greater size of *M.cornuarietis* compared to other studied gastropod molluscs (such as *B.glabrata* and *Lymnaea stagnalis)* resulted in greater hemocyte yields and it was also much easier to handle. If *Marisa* hemocytes can be shown to have similar morphology, physiology and function to *B.glabrata*, then this species could be a useful model when assessing immune function in gastropods due to its practical advantages. *B.glabrata* display considerable variation between individuals in both hemolymph yield and hemocyte response and it is possible that *M. cornuarietis* may respond more predictably than *B.glabrata. B.glabrata* is by no means the perfect model mollusc species, but happens to be the most medically important. Nevertheless, *Marisa* is of medical importance to humans due to its role in ecological systems, as a biological control of vectors of disease, including *B.glabrata*.

If *M. cornuarietis* could be shown to be a more reliable test species than *B.glabrata* it may be useful in preliminary studies or for assay refinement, in the same way that techniques are often pioneered in rats and mice before use on humans. As both *B.glabrata* and *M. cornuarietis* are invertebrates, the latter offers no research advantages in terms of animal experimentation ethics, but could provide a means of establishing mollusc-based immunological assays in a less variable and labour intensive species than *B.glabrata*. There are currently no studies reporting the use of *M.cornuarietis* as a model mollusc for *in vitro* immunological studies so this work may help to form the beginnings of such research.

2.4.2. Distribution

Marisa cornuarietis is endemic to areas of central and Northern-South America in the countries of Bolivia, Brazil, French Guiana, Suriname, Guyana, Colombia and Venezuela. It also has a distribution in the Central American countries of Panama and Costa Rica and in Trinidad and Tobago (Pastorino and Darrigan, 2011; Figure 2.8). In general, its distribution is very similar to that of *B.glabrata* as are its habitats. These primarily include stagnant and slow running freshwater but not temporal water bodies as the eggs must remain submerged (Pastorino and Darrigan, 2011).

Marisa snails are natural competitors and predators of *B.glabrata,* feeding on their eggs, hatchlings and even on adults (Ruiz-tiben, 1969). For this reason, *M.cornuarietis* has received considerable attention as a means of biological control against *S.mansoni via* its negative impact on the parasites primary vector (Selck *et al*., 2006). Populations of *B.glabrata* were successfully reduced in transmission areas of Puerto Rico after the introduction of *Marisa* (Jobin, 1970). This species has also been used for biological control in the context of invasive aquatic plants as its large size means it is capable of eating considerable amounts of vegetation (Robins, 1971).

Due to its use as a biological control agent and popularity as an aquarium species it also has established itself as an invasive species in some unusual locations far from its native areas. It was first discovered in North America in the state of Florida in 1957 and subsequently in Texas, California and Idaho (Rawlings *et al*., 2007). The most recent report of *M.cornuarietis* as an invasive species comes from Spain where

the snail was found inhabiting a natural freshwater ecosystem in the north of the country (Arias and Torralba-burrial, 2014).

Figure 2.8. The global distribution of endemic and invasive *M.cornuarietis. Areas with endemic distribution are shown in blue and invasive distribution shown in red.*

2.4.3. Biology

Marisa, often referred to as the 'Giant ramshorn snail' is a gastropod genus belonging to the family *Ampullariidae* (the Apple snails). Despite being termed 'Ramshorn snails' they are not related to the *Planorbidae* (or 'true ramshorns') which include *Biomphalaria*. The genus *Marisa* contains two known species; *Marisa planogyra* and *Marisa cornuarietis*, the latter being the better studied of the two (Rawlings *et al*., 2007).

These snails are among the largest freshwater gastropods, with shell diameters often exceeding 50mm (Selck *et al*., 2006). The *Ampullariidae* possess both a clearly defined lung and a monopectinate gill, making them effectively amphibious (Schirling *et al*., 2006). Unlike *Biomphalaria*, the *Ampullariidae* are gonochoristic,

with individuals being exclusively either male or female and they also have an operculum which acts as a protective lid covering the entrance to the shell.

Although laid underwater, the eggs of *Marisa* do not resemble those of Planorbids, in that they are laid in a large, gelatinous, mass not unlike frog spawn, which sticks to aquatic plants and consists of between 20 to 80 spherical eggs of around 5mm in diameter (Schirling *et al*., 2006). The gelatinous material also serves as food for the developing snails, which will hatch after around 12 days at 26ºC (Sawasdee and Kohler, 2009). Snails reach sexual maturity at around 6 months of age and females will usually first spawn after around 8 months (Janer *et al*., 2006).

The hemolymph of *M.cornuarietis* is typical of most molluscs, in that its respiratory pigment is hemocynin and subsequently the hemolymph is pale blue in colour, unlike the red hemolymph of *B.glabrata* (Chapter 1, Figure 1.9).

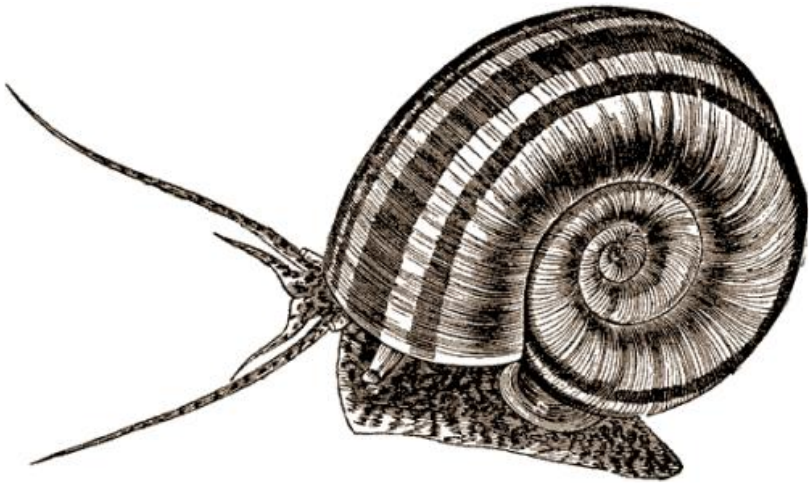


Figure 2.9. Illustration of *Marisa cornuarietis.* Original image drawn by William Charles Linnaeus Martin (1844). Available: http://www.gbif.org/species/2292586 [Accessed: Jan 23rd 2015].

2.4.3.1. Immunology

M.cornuarietis is generally not considered to be of medical importance as a disease vector. Consequently, very few studies have investigated its immune system. It has been shown to support experimental infection with species such as *Angiostrongylus cantonensis*; a parasitic nematode which can cause potentially fatal eosinophilic meningitis in humans (Yousif and Lämmler, 1975).

Yousif and Lammler (1975) tested sixteen species of aquatic snails from four different families and found that all could support a transmissible infection, but that the rate and intensity of infection varied considerably. *M. cornuarietis* showed a significantly more vigorous immune response to *A. cantonensis* and gave rise to fewer viable larvae than did *B. glabrata* (Yousif and Lämmler, 1975). Since the level of compatibility of gastropods to infection with *A. cantonensis* shows such a wide degree of variation between species, it was suggested by Harris and Cheng (1975) as an ideal parasite-mollusc model for comparative studies on cellular responses which are not possible with other parasite species (e.g. *S. mansoni*) due to the limited number of species that can support an infection.

As a gastropods, histological studies have shown that the immune responses of *M. cornuarietis* to parasitic infection are similar to those of *B.glabrata*. The primary response to parasites is encapsulation, to varying degrees of intensity, with defence cells seen to flatten over the larvae (Yousif *et al.,* 1980). The cellular reactions against the larvae were found to increase with time and numerous larvae can be seen to have degenerated, with reactions around the degenerated larvae being stronger than around those still intact (Yousif *et al.,* 1980).

While no serious attempt has been made to classify the hemocytes of this snail, current research suggests two principle types of hemocyte and these usually follow the same fundamental classification as for *B.glabrata* i.e. cells which spread and those which don't (Yousif *et al*., 1980).

M.cornuarietis is also thought to possess an amoebocyte-producing organ, based on the identification of a band-like region of cells which was enlarged and more active in infected snails (Yousif *et al.*, 1980).

2.4.4. Use in ecotoxicology

For a long time, invertebrates were believed to be too phylogenetically distant from humans to be of any use as models of human disease research. We now know that several species which are morphologically extremely different from one another can actually share relevant pathways determining the development of cancers and even dementia-like symptoms (Perkins *et al*., 2013). Indeed, the fundamental pathways

determining reproduction, development, stress response and innate immunity are highly conserved among metazoans, allowing for a degree of extrapolation to higher species (Perkins *et al*., 2013). Cell models, such as those relating to gastropod hemocytes and even neural cells, have facilitated our understanding of the fundamentals of more complex processes in mammals (Yoshino *et al.*, 2013). This has led to an increased desire to discover other 'model species' that could be used in preliminary studies, while reducing our reliance on vertebrates with their associated logistical, financial and ethical constraints (Guilhermino *et al*., 2000).

Molluscs are an extremely diverse phylua and even individuals that appear morphologically similar may be more genetically dissimilar than any two vertebrates. As such, they offer considerable choice and challenge as possible model species.

A number of studies performed in the last two decades suggest that certain species of molluscs are adversely affected by compounds that act like endogenous vertebrate-type sex steroids (Ketata *et al.*, 2008). If this is the case, it would enhance the profile of genera like *Marisa* as substitute model organisms for the study of feminization and intersex mechanisms relating to vertebrates. However, the endocrine system of molluscs differs in many aspects to those of vertebrates and sex-steroids, if produced, may not have the same function. Indeed, there is still much controversy in this area (Dietrich *et al*., 2006; Ketata *et al*., 2008; Scott, 2013).

Several studies have directly investigated the use of *M.cornuarietis* as a substitute to vertebrates, particularly the zebrafish (*Danio rerio*), in toxicology testing. The *Danio rerio* embryo toxicity test (DarT) is a well-established method for screening developmental, morphological and a range of other sub-lethal and lethal effects on developing fish (Hanisch *et al*., 2010). The popularity of this method is due in part to the lighter regulation regarding embryos, their comparatively higher numbers and lower space requirements compared to adults (Hanisch *et al*., 2010). Due to the superficial similarities between the eggs of *Marisa* and zebrafish (size, circularity and transparency) a test was developed to use the snail eggs/embryos as an alternative for toxicological testing (Schirling *et al*., 2006). Endpoints included tentacle and eye formation, heart rate and hatching success. The *Marisa* embryo toxicity test (MariETT) has since been adopted by several groups and tested against

compounds including lithium, copper, atrazine and imidacloprid (Osterauer *et al*., 2009; Sawasdee and Kohler, 2009; Sawasdee and Kohler, 2010). On the basis of lowest observed effect concentrations (LOECs), Sawasdee and Kohler (2009) reported that the MariETT was three orders of magnitude more sensitive to tested metals and at least one order more sensitive to biocides, than the *Danio rerio* embryo test.

In addition to the mechanisms already mentioned, many of the elements of the molluscan defence system are functionally very similar to those of mammals and indeed, both share an innate system, the function of which has been very well conserved thought evolutionary history (Ottaviani, 2004). For this reason, invertebrates such as *Marisa* could also be considered as an alternative to vertebrate immnuotoxicology testing.

It is also important to remember that substitution for mammals is not the only incentive for considering invertebrates as model organisms. Certain invertebrates can also be considered as alternatives, or models, in place of other invertebrates. For example, species such as *Biomphalaria glabrata* are of considerable research interest due to their medical importance, but have limitations in their use due to their often poor hemocyte yields and/or responsiveness.

2.4.5. Husbandry

Since *M.cornuarietis* is not currently a widely used test organism, relatively little has been established regarding its husbandry compared to *B.glabrata* (Forbes *et al*., 2008). Due to the increase in interest regarding *Marisa*, at least two recent studies have attempted to investigate the optimal laboratory rearing conditions (Aufderheide *et al*., 2006; Selck *et al*., 2006).

2.4.5.1. Water temperature

In molluscs, it is well established that metabolism and growth increase with increasing temperature and Akerlund (1969) demonstrated this for *Marisa* over a range of 20-35^{o}C.

35ºC is typically considered to be too high for these snails in terms of increased mortality and stress and is quite far from their naturally experienced average temperature. Snails have been shown to grow at a significantly faster rate when increasing the temperature from 25ºC to 28ºC and 28ºC is still within 'normal' limits (Aufderheide *et al*., 2006). Aufderheide *et al*., (2006) found no differences in egg size or numbers between snails grown in the range of 22-28ºC, however at 22ºC egg hatchability was reduced by 72% and those which hatched took 42% longer to do so. 22ºC appears to be the lower limit of the acceptable range for a constant temperature as at this point developmental rates begin to slow significantly and mortality rates were almost twice as high and growth half as fast when compared to 25ºC (Selck *et al*., 2006).

Aufderheide *et al*., (2006) concluded that 25°C–28°C lies centrally within the tolerance range of *M. cornuarietis*. In keeping with these findings we maintain our snails at 27ºC which is conveniently also around the optimal temperature for *B.glabrata*, though not surprising due to their geographic overlap.

Within the optimal temperature range, snails are expected to reach a shell diameter of 20mm (the point at which sex can be visibly determined) at around 89-101 days post-hatch and 30mm at around 127-145 days (when sexual maturity is reached) (Aufderheide *et al*., 2006).

2.4.5.2. Light

Compared to seasonal breeding species in which photoperiod is an extremely important signal for reproduction, the role of light reproduction in tropical species is less clear.

Aufderheide *et al*., (2006) found that alterations in photoperiod had no influence on fecundity traits or egg-hatching but interestingly, juvenile snails grew significantly faster at 28°C than at 25°C when the 24-hour photoperiod was 12 hours of light and 12 hours of dark (12L:12D), but no difference was observed at 16L:8D.

My snails were kept under a 12h light; 12h dark cycle, which was optimal and also fits with that of *B.glabrata*, meaning the two species could be housed in the same room.

2.4.5.3. Density

Due to its large size, densities for *M. cornuarietis* should be kept lower than for most other aquatic gastropods. Just less than 1 snail per litre (0.8 per L) has been reported as optimal with densities of 2 snails per litre or higher causing a significant reduction in growth, frequency of egg laying and rate of hatching success (Aufderheide *et al*., 2006). The mechanisms behind density dependence have not been investigated in *Marisa* but are likely to be similar to those for *B. glabrata*. My snails were kept in flow-through tanks with densities never exceeding 1 snail per litre.

2.4.5.4. Food

In the laboratory *Marisa* has been successfully fed a variety of foods including lettuce and algae, but snails are reported to have a preference for higher-protein diets (Hofkin *et al*., 1991). Not only nutrient composition but also digestibility are important and for this reason we fed our snails the same TertraMin® as for *B.glabrata* (Selck *et al*., 2006).

In my experience the optimal husbandry for *M.cornuarietis* very closely matches that of *B.glabrata*, probably because both are aquatic gastropod molluscs and share a very similar geographic distribution. This means that few, if any, additional requirements were necessary with respect to laboratory husbandry, which could potentially encourage departments that already culture *B.glabrata* to also investigate this species.

Chapter 3: *Assay validation*

3.1. Test chemicals

Given my hypothesis; that the host-parasite relationship is affected by immunomodulatory effects of pollution, I selected 3 representative compounds that are amongst the most commonly reported freshwater organic pollutants in *S.mansoni* transmission areas (Table 3.1), as well as throughout much of the world. In the interest of diversity, each of these compounds is from a different class of pollutant; an endogenous hormone, a plastics component and a pesticide. In addition to these compounds I also tested an extract of primary-treated urban effluent, which would serve as a more environmentally relevant mixture of compounds.

3.1.1. 17β-estradiol (E2)

Steroid estrogens can be categorized into two main groups: natural and synthetic. Major natural estrogens include estrone (E1), 17β-estradiol (E2) and estriol (E3), which are endogenously produced by all vertebrates, including humans, and possibly in some insects (Mechoulam *et al*., 2005; Limpiyakorn *et al*., 2011). Synthetic estrogens, such as 17α-ethinylestradiol (EE2), are typically analogues of natural estrogens like E2 and are created by pharmaceutical companies for purposes such as birth control (Limpiyakorn *et al*., 2011). Both natural and synthetic estrogens are excreted in urine and faeces and distributed into the aquatic environment *via* sewage discharge, animal waste and soil run-off (Limpiyakorn *et al*., 2011). 17β- estradiol (E2) is the strongest, most active, and most widely distributed of the estrogens found in the environment and is therefore one of the more problematic pollutants, especially due to its endocrine disrupting effects in aquatic wildlife (Jobling *et al*., 2005; Ruan *et al*., 2014).

A third group of estrogenic chemicals are the so-called xenoestrogens; chemicals which are not created by the body, nor are they synthesised to have estrogenic activity, but, due to their structure, happen to possess estrogenic properties nonetheless (Fang *et al*., 2001). The number of chemicals which act as xenoestrogens is considerable. Indeed, all the chemicals used in the following work are known xenoestrogens, despite being used for very different purposes.

3.1.2. Bisphenol A (BPA)

Perhaps the best know xenoestrogen, bisphenol-A (BPA), is an organic compound widely used as a monomer in synthesis of epoxy resins, polycarbonate plastics, polyacrylates and polyesters (Sharma *et al*., 2009). Due to its diversity of uses, BPA is one of the most highly produced chemicals worldwide, with more than 3.6 million tonnes produced in 2011 (Rubin, 2011). BPA enters the aquatic environment primarily through wastewater effluent but is also known to leach from plastic products floating in the water (Kang *et al*., 2007)**.** BPA-induced endocrine disruption has been shown in many vertebrate classes including mammals, fish and amphibians (Canesi and Fabbri, 2015).

3.1.3. *p'p*-Dichlorodiphenyldichloroethylene (DDE)

One of the best known and earliest studied environmental contaminants is the organochloride pesticide DDT (dichlorodiphenyltrichloroethane). Due to its adverse environmental impact, DDT is now banned in most countries, although its use is still permitted in some areas for the control of malaria-vector mosquitos (Gerić *et al*., 2012). Due to the persistent nature of DDT, its ability to biomagnify and its extensive use prior to the ban, DDT and its breakdown products are still present in aquatic systems around the globe (Gerić *et al*., 2012). The primary metabolite of *o,p*-DDT is *p'p*-Dichlorodiphenyldichloroethylene (DDE), which typically persists much longer than the parent compound and is believed to be responsible for the majority of the negative effects associated with DDT exposure (Binelli *et al*., 2008; Arrebola *et al*., 2013).

3.1.4. Effluent extract

The term 'effluent' refers to the water that is discharged from a treatment plant into the aquatic environment. Effluent represents the main source of xenobiotic substances in most of the world's water bodies. Since effluent is a complex mixture of thousands of different chemicals, as would occur in the environment, it arguably represents the most environmentally relevant substance with which to test the effect

of pollution in experiments; for this reason, an effluent extract was chosen as a test chemical along with E2, BPA and DDE.

The extract was made from a 1 litre, 24-hour composite sample of primary effluent, which was collected from a conventional activated-sludge plant in London. Primary effluent is sewage which has been removed of suspended and floating solids *via* gravitational sedimentation and represents the most basic form of treatment.

On the same day as the effluent sample was collected, solid phase extraction was performed using a C18 disk (47mm diameter, Empore™) to bind the organic chemicals. Chemicals bound to the disk were eluted with methanol, which was subsequently evaporated and replaced with 1 ml of absolute ethanol, resulting in a 1000x concentrated effluent stock. A more detailed explanation of the effluent extraction process can be seen in Chapter 7.

3.2. Doses

The nominal dose ranges selected for the *in vitro* assays covered a relatively broad spectrum of concentrations since levels found in the environment differ substantially by location and season. Also, the use of concentrations that are higher than those found in the environment might compensate for the lower uptake of cells *in vitro* compared to tissue concentrations in a whole animal, which are often considerably higher than environmental concentrations (Gadd, 2000; Groothuis *et al*., 2015). To achieve concentrated stock solutions ethanol was used as a carrier solvent, with final concentrations not exceeding the maximum limit according to OECD guidelines (OECD, 2009). Control doses (i.e. those containing no test-chemical) contained a percentage of ethanol equal to that of the rest of the doses to account for possible solvent effects.

Table 3.1. Studies which have reported some of the high-levels of my chosen test-chemicals in freshwater-bodies of *S.mansoni* transmission countries. *These example were chosen to give an indication of the higher environmental levels that host snails could feasibly be exposed to.*

Chemical	High dose (µg/L)	Location	Reference
DDE	90	Saudi Arabia	(El-Saeid *et al*., 2011)
E2	0.062	Brazil	(Moreira *et al*., 2011)
BPA	3.61	Brazil	(Melo and Brito, 2013)

3.3. Statistical Approaches; Methodology and Rationale

Several of the experiments described in this thesis take the form of a dose-response, hence the need to discuss some of the pitfalls commonly encountered with this type of analysis, the ways they can be avoided, how to deal with more complex dose-response relationships, situations where the correct decision is ambiguous and also times when it may be justifiable to deviate from the 'ideal'.

3.3.1. The dose-response relationship

It is typically asserted that an important consideration in judging whether an association between a chemical and a biological reaction is causal is the presence, or absence, of a dose–response relationship (Rosenbaum, 2003).

The dose–response relationship describes the change in an observable function or behaviour of an individual organism, or a population, induced by differing levels (doses) of exposure to a stressor, usually a chemical (Altshuler, 1981).

The analysis of variance (ANOVA) test has been heavily used for the analysis of biological, behavioural and psychological data since its inception nearly 100 years ago by the pioneering statistician Ronald Fisher (Fisher, 1925; Cottingham *et al*., 2005). The popularity of this method probably stems from its relative simplicity and flexibility in terms of computation and interpretation, which means that it is one of

the first statistical methods taught to students and is widely availability in statistical software.

ANOVA is an appropriate statistical test in many situations, but is not ideal for use in dose-response assays. Despite this, its use as the primary means of analysis in such studies is still extremely common.
In reality ANOVA and regression, while often taught as being two entirely separate types of analysis, are in-fact almost exactly the same, the only real difference being how we treat the data (Field, 2013).
While ANOVA is effective at detecting the existence of a dose-induced response (discussed below), it cannot describe the actual shape or magnitude of the relationship because it does not consider the order of the doses; a vital component of the dose-response assay (Zhu *et al.*, 2005). This is because ANOVA tests assume that the independent variable is *categorical* i.e. not of a *continuous* and meaningful order. Increasing chemical dose however, is a *continuous* variable and the order of doses is extremely important (OECD, 2010).

The use of an ANOVA for this kind of data is not necessarily 'wrong' since it can be used effectively, if appropriately, in the context of these types of experiment but never to understand the actual dose-response relationship, which is often the main point of the experiment. The ANOVA does not provide information regarding the type of relationship, as a curve would, but indicates which doses differ from one another in the response they elicit. Despite this, it is often apparent that many studies 'shoehorn' dose-response data so that it will fit into an ANOVA. This process of turning a continuous variable into a categorical one is known as dichotomizing (Drummond and Vowler, 2012). An example might be classifying the dose into categories of 'low' or 'high' thus creating a 'cut-point' when in reality there is no true definition of high or low dose, since all the doses lie on a continuous spectrum, in the same way there is no definition of 'old' or 'young' since age is a continuous variable. By doing this, doses which differ by one nanogram could be classified as different when they are likely to be very similar (Sauerbrei and Royston, 2010). Dose-response relationships may not be linear but would certainly be expected to be smooth, something which is not possible to show when using categories.

3.3.1.1. How to investigate dose-response relationships

Regression-based tests answer the question of how the response variable is affected by changes to the independent variable(s) by building a model to attempt to describe the shape of the relationship between (X) dose and (Y) response – can we predict Y if we know the value of X? (Cottingham *et al*., 2005).

The most basic models are those of simple linear regression when, for example, the relationship might be best described by a straight line running through all the data points. Such a model might be defined as y = a + bx where 'a' is the y intercept and 'b' the slope (Rumsey, 2007).

For many years, toxicologists relied on the presumption that increasing dose of a chemical will result in an overall linear response with the direction dependant on the type of chemical i.e. antagonist or agonist (Figure 3.1A and B). In the case of ecotoxicology, where the study chemical is a 'pollutant' which is expected to have a negative impact, this would typically mean that the higher the dose the greater the harm (Schug *et al*., 2011). In reality such relationships are not likely to be perfectly linear and while described as such, they are more likely to take on the appearance of a smooth, consistent, curve which is known as monoticity (Figure 3.1C). This type of model is traditionally used by regulators to establish risk assessment profiles for chemicals, with risk being proportional to dose (Schug *et al*., 2011).

These concepts hold true for many chemicals but more recently it has become widely accepted that deviations from monotonicity (non-monotonicity) not only occur, but are in fact common for certain classes of chemical (Davidson *et al*., 2006).

In biological systems there are typically numerous factors/mechanisms acting at the same time to control a single response variable. This can mean that the exposure variable may act differently on individual mechanisms, which can result in a nonlinear relationship (Bhujel, 2008). Homeostatic mechanisms can often produce nonlinear dose-response relationships due to the fact that they are working to maintain normality. If a chemical disrupts a process causing the curve to go up or down the homeostatic mechanism attempts to counteract the effect, causing the curve to change direction (Beckon *et al*., 2008; Myers *et al*., 2009).

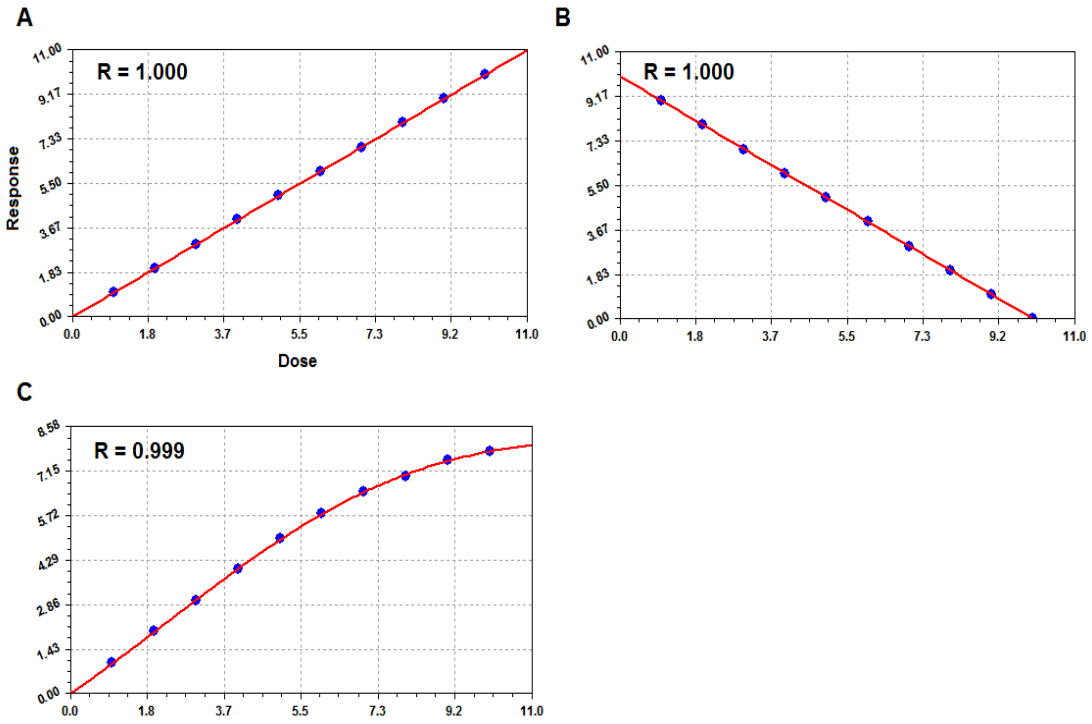


Figure 3.1. Hypothetical examples of strong linear regression relationships seen in dose-response assays. *A) Perfect positive linear relationship B) Perfect negative linear relationship C) A more realistic strongly positive relationship. R = correlation coefficient.*

Non-monotonic dose-response (NMDR) curves can take numerous shapes but are characterised by the fact that they do not follow a consistent increase or decrease over the tested range but instead may bend up and down. For example, an NMDR may show stimulatory responses at the beginning and the end of the dose range with a 'dip' in the middle (Colborn *et al*., 1997; Lagarde *et al*., 2015). These shapes are commonly referred to as 'U' or 'inverted U'.

The group of chemicals which have received by far the most attention regarding their tendency toward non-monotonic dose-responses are endocrine disruptors (Vandenberg *et al*., 2012). EDCs are capable of eliciting bi-phasic dose-responses for many different endpoints, at many levels of organization, such as cell proliferation and organ development (Schug *et al*., 2011). The existence of NMDR relationships puts into question the adequacy of the traditional testing paradigms and highlights the need to use appropriate models to adequately describe the relationship (Schug, *et al*., 2011).

3.3.2. Beyond simple linear regression

Due to the more complex nature of the dose-response relationship invoked by certain chemicals, high-order models are often more appropriate in order to accurately model them (Smyth, 2002).

One major family of models used to describe NMDRs are the polynomials (Rumsey, 2007). A first-order polynomial model is a straight line, a second-order forms a parabola shape similar to the 'U' often seen in NMDR curves and third-order polynomials change direction twice, something also relatively common to NMDR curves (Rumsey, 2007). There are higher-order polynomial models but a rule of thumb sometimes used in biological models is that if the data can't be fit by the time the polynomial model reaches a third order then the relationship is ambiguous (Rumsey, 2007).

It is technically possible to fit a model perfectly to any data set, by simply joining the data points together the model would perfectly describe the data; this raises two important issues with complex regression models; (i) the value of R^2 as a definitive indicator of how well the model fits the data and (ii) computer modelling and over-fitting.

3.3.3. R^2 and over-fitting

It is common practice to assess the fit of a model by simply looking at the value of R^2. This value indicates how closely the curve fits the data, but may be misleading due to over-fitting. The purpose of regression analysis is to provide a realistic description of the true relationship between the two variables and also to extrapolate values for Y based on unknown values for X. R^2 does not tell us whether the relationship is 'realistic'; this can only be done by visual observation.

Due to the complex nature of some of the models required to describe certain kinds of data, including EDCs, curve-fitting software can be used to aid model selection. Such software has numerous pre-defined linear and nonlinear regression models and matches the data against each to find the best fit. The 'best' models are then ranked on the strength of the R^2 value alone, something which is often achieved by over-fitting data using high-order polynomial models with excessive numbers of variables

to force the curve through the points (Figure 3.2A). I used curve-fitting software (CurveExpert 2.2.0) to aid selection by looking at the graphs and choosing the most 'sensible' model which fits the data and could be reasonably expected, given the experimental design and hypothesis (Figure 3.2B). The final regression models and graphs were created in Graphpad Prism 6.0.

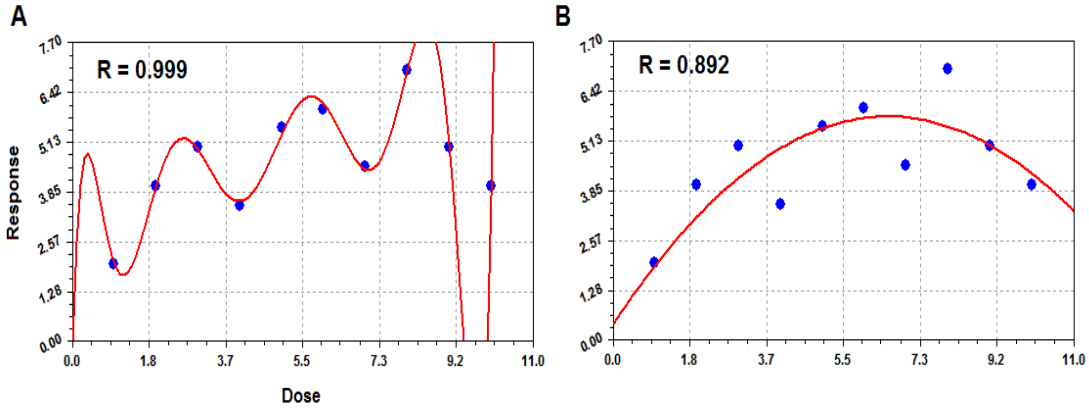


Figure 3.2. Over-fitting demonstrated by the best-fit regression model for a hypothetical data set. *Regression model according to A) software (CurveExpert Pro) and B) visual inspection.*

There are further ways to ensure that the potential for over-fitting is reduced. Before running the curve-fitter, the maximum number of variables to include in the model can be set, in my case this would typically be 3 i.e. a third-order polynomial, which is still feasible given that we are dealing with EDCs. The other step that can be taken is to only report the adjusted R^2 value rather than the standard R^2. The adjusted R^2 equation penalizes the addition of extra variables to the model, which may be of very little relevance in explaining the true relationship but still inflate the R^2 value to an artificially high level. The adjusted R^2 value therefore represents a more conservative estimation and is especially useful when dealing with polynomials (Rumsey, 2007).

In his authoritative work entitled 'Statistical Power Analysis for the Behavioural Sciences', Cohen (1988) outlined a number of criteria for gauging small, medium and large effect sizes based on r^2 value:

r effects: small ≥ .10, moderate ≥ .30, large ≥ .50

Throughout the thesis this criterion will be used as a means by which to describe the relative response of a regression relationship.

3.3.4. Adjusting variable data

A common method in dose-response studies is to apply a logarithmic transformation to the X (dose) values. Since doses are typically spaced in orders of magnitude, the data points are positively skewed with several data points at the beginning of the dose-range grouped much closer together than those at higher doses, making the graph difficult to interpret. The transformation results in equal spacing between data points to aide with visualization but has no influence on the best fit values or their standard errors or confidence intervals (Motulsky, 2003).

Another common practice is to perform analysis on the mean value of Y, that is to average the value of independent replicates of Y for each value of X and then to enter a single data point into the software for analysis. The result is that the sum-of-squares will be much smaller, the points will be closer to the curve on average and the R^2 much higher than if all the replicates were analysed independently. Table 2.4 shows the difference in adjusted R^2 value from the same data set when the individual results are entered (adjusted $R^2 = 0.5276$) and when only the means are entered (adjusted $R^2 = 0.8167$). Often the data are only displayed graphically as single data points (the means) for simplicity, but the analysis should always be performed on all the independent replicates.

Table 3.2. A hypothetical example to demonstrate the difference in adjusted R^2 values when analysing individual data points or mean values for Y. *Analysing mean values results in an inflated Adjusted R2 value (0.8167) when compared to analysing data points individually (0.5276).*

X	**Y:A**	**Y:B**	**Y:C**	**Y:mean (A-C)**
1	1.7	1.411	1.93	1.680
2	1.294	1.62	1.49	1.468
3	1.3	1.21	1.79	1.433
4	1.396	1.37	1.2	1.322
5	0.986	0.88	1.06	0.975
Adjusted R^2:	**0.5276**			**0.8167**

Where regression models are used in my studies, each data point is derived from at least 3 independent replicates of the Y value with each one entered into the model individually and not averaged *a-priori*.

3.3.5. Ambiguous instances

Count data can be ambiguous in its definition and distinction between how it is classified as a variable and subsequently the way in which it is treated (Gotelli and Ellison, 2004). McDondald (2014) gives the following example: If a researcher were counting the number of bacterial colonies on a plate e.g. 87 on the first plate, 92 on the second etc., then each plate would have one data point and therefore be a measurement variable. If instead the aim was to count different coloured bacterial colonies on each plate e.g. 'red' and 'white' this would be a nominal/categorical variable since each colony has a separate data point with 2 possible categories of red or white. A further point of confusion that often appears in the literature stems from the fact that in the later example, where data are categorical, it is common to summarize the results as a percentage e.g. percentage of red colonies verses white colonies. While this is a perfectly reasonable way of displaying the data it is often treated as continuous due to the numerical value, even though the underlying data remains categorical.

3.3.6. Where is it acceptable to deviate from the ideals?

There are circumstances where it is justifiable to deviate from the preferred methods described above. Logistical constraints, properties of the study chemical and sample size are some examples. One of the most likely reasons that the ideal analysis may not be achievable is due to the variability and non-normal nature of the biological system and/or response being studied. Certain types of response are recorded as variables which do not suit standard dose-response analyses that assume a continuous response/dependent variable. Count data, for example, is often highly positively skewed due to larger numbers at low counts and therefore might violate the assumptions of preferred methods. Methods to optimize this type of data are often complex and not widely available in software packages and/or without expert

advice. An example of an appropriate model for count data may include zero-inflated Poisson, although some authors question the necessity of such approaches given their complexity (Allison and Allison, 2012). In situations where the data is highly positively skewed several authors state that dichotomizing becomes a valid option since the disadvantages of this method are outweighed by the problems caused by dealing with the data in a regression model (MacCallum *et al*., 2002; Streiner, 2002). Another option for allowing such data to be used in dose-response analysis would be to perform a transformation to allow for normal distribution and some researchers prefer this approach over dichotomizing (MacCallum *et al*., 2002; Crawley, 2005).

3.3.7. ANOVA and multiple comparisons

Despite the limitations of treating continuous data as categorical in dose-response studies (false precision and loss of information) and the advantages of regression/polynomials, several authors have pointed out the potential for using categorical variables for purposes of exploration and to yield important insights (Motulsky and Christopoulos, 2004; May and Bigelow, 2005).

While ANOVA-type analysis has been considered by some as a 'fall-back' when data fail to meet the criteria for regression, it has also been suggested that the relative advantages of both could be combined (Isnard, *et al*., 2001; Motulsky and Christopoulos, 2004; Cottingham *et al*., 2005; Berger, 2010).

If the initial ANOVA is significant then differences exist between the dose and the response, but where the difference lies is unknown (Hau and Schapiro, 2003). To find out where the differences lie it is possible to use a multiple comparisons test on group means as *post-hoc*. The preferred test in the context of dose-response is Dunnett's test which compares the response at each dose to the response of the control. Another relatively common multiple comparisons test is Tukey's HSD which compares every combination of dose and response.

Multiple comparisons are not without drawbacks. Testing multiple comparisons raises the probability of making a type 1 error since it is akin to 'fishing' for significance. In a similar manner to adjusted-R^2 in regression, multiple comparisons can be controlled for type 1 error by performing corrections on the p value (Souba

and Wilmore, 2001). Also like the adjusted R^2, the corrections result in a loss of power. However, being more conservative is usually preferred.

The approach of performing regression when appropriate, followed by multiple comparisons tests is one that I have adopted for dose-response studies as it yields the most amount of useful information i.e. the shape of the relationship, the ability to extrapolate missing values and also the identification of doses which are significantly different. My approach was similar to that used by McMahon *et al*., (2011).

3.4. Validation experiments

The following section outlines the assays which were performed in-order to help determine that the desired end-points were actually being measured as opposed to potential confounders.

3.4.1. Dose-range effects of test chemicals on hemocyte viability

Given that my studies are concerned with measuring alterations in hemocyte immune-responses and not direct cytotoxicity, it is important to test the effect of the chosen chemicals, across the chosen dose ranges, on hemocyte viability. Without establishing whether chemical doses result in reduced viability we cannot establish to what extent any subsequent observed reduction in immune-response is actually a result of cells failing to respond due to toxicity, as opposed to subtler and non-lethal changes in behaviour and function of cells, resulting in suppressed immune responses.
The aim of my work was not to determine the lethal levels of the chosen pollutants on hemocytes since, from an ecotoxicological perspective, this would have limited relevance as an organism or cell is unlikely to ever be exposed to such large quantities in a natural setting.

For each replicate, 100µl pooled hemolymph was collected as described in Chapter 2 and mixed with an equal volume of CBSS, containing the test chemical, then

transferred to wells of a 24-well plate. After incubation for 1-hour at 27ºC, the majority of hemocytes had spread to form a monolayer. The supernatant was removed from the monolayer and replaced with 100µl α-CC and incubated for a further 10 minutes. After the final incubation an equal volume of 0.4% trypan blue was added to the well and the solution mixed. Immediately after mixing, a 10µl sample was taken and added to a hemocytometer where 100 cells were assessed for exclusion or incorporation of the dye. This process was repeated three times for each experimental replicate of each dose.

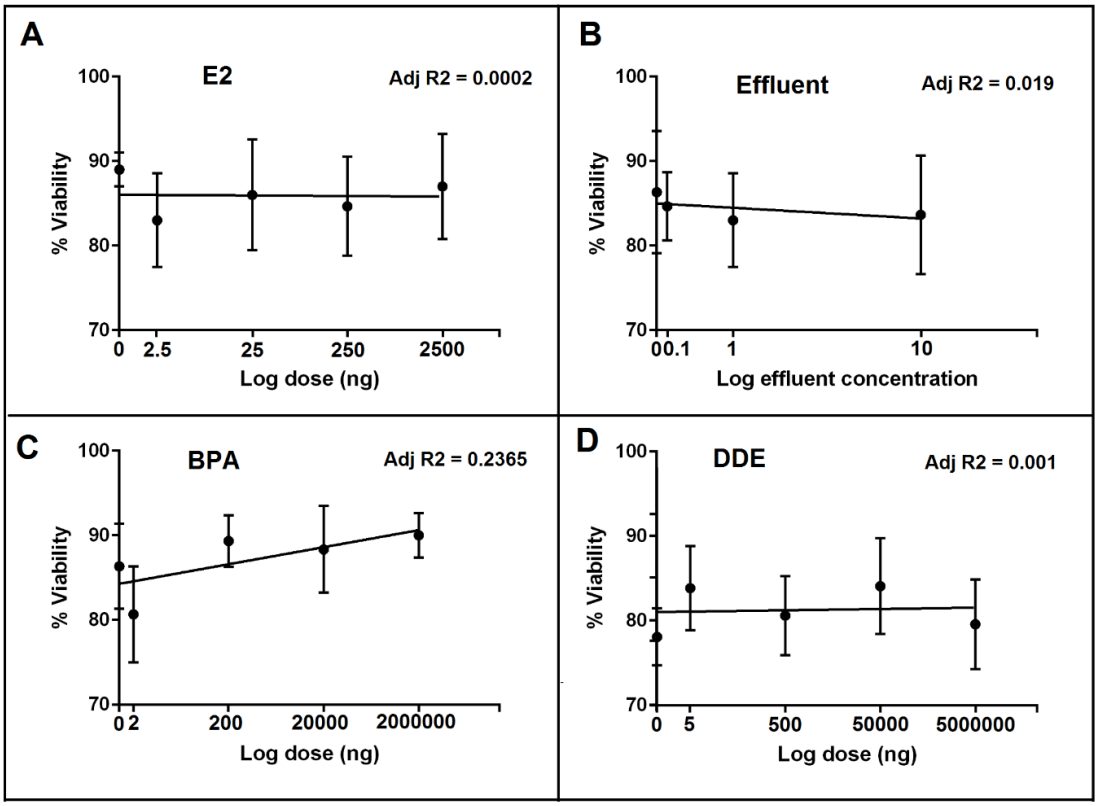


Figure 3.3. Dose-response assays used to determine whether significant toxicity occurred to hemocytes across my chosen dose range for each test chemical. *A) E2 B) Effluent extract C) BPA D) DDE, hemocyte viability measured as % of viable cells (± SEM of 3 independent replicates) according to Trypan blue exclusion.*

None of the test chemicals showed any meaningful relationship between increase in dose, within the measured range, and reduction in cell viability according to the trypan blue exclusion assay (Figure 3.3A-D). A very slight positive relationship appeared for BPA (Figure 3.4C) but there is no existing work which would suggest that this is anything more than natural variation. Indeed, viability testing proved to be the most variable of the assays which were performed. Molluscan hemocyte

populations are known to show very high degrees of difference in viability between individuals, in my study this variation could be a residual effect of having pooled cells from different snails, leading to a more heterogeneous population (Ashton-Alcox and Ford, 1998).

3.4.2. Inhibitor dose-response for *in vitro* assays

To determine if chemically-induced alterations in the immune-response of interest can reliably be measured, it was important to include compounds with established and reliable effects which are not due to direct cytotoxicity. Sodium azide (NaN_3) is known to be a potent cytochrome C inhibitor in many different cell-types from numerous species, including molluscan hemocytes (Goedken *et al*., 2004). Inhibition of cytochrome C results in the inhibition of ATP-synthesis, ATP being the main source of energy for the majority of cellular functions (Sun *et al*., 2010). Cellular functions dependent on ATP, and thus shown to be inhibited by sodium azide, include phagocytosis, motility and aggregation (Thomas *et al*., 1992; Goedken *et al*., 2004).
In studies involving molluscan hemocytes sodium azide, at concentrations of up to 2%, has been shown to considerably reduce phagocytosis without significant reduction in cell viability (Brousseau *et al*., 2000; Sauvé *et al*., 2002).

Figure 3.4 shows that the response of each immune-endpoint (Figure 3.4; A, B and C) is strongly suppressed with increasing doses of NaN_3, while hemocyte viability is minimally reduced across the same dose range (Figure 3.4; D). These results are in accordance with previous reports on the ability of NaN_3 to suppress hemocyte function at similar concentrations, without inducing significant loss in viability (Sauvé *et al*., 2002; Goedken *et al*., 2004). The importance of obtaining this type of preliminary data can be demonstrated using cell motility as an example. If we were to see a reduction in mean cell velocity with increasing dose of NaN_3, but did not know that NaN_3 was cytotoxic within the dose range used, then it could appear that reduction in mean velocity was due to sodium azide-induced inhibition, when in reality the decrease was as a result of the death of individual cells within the population.

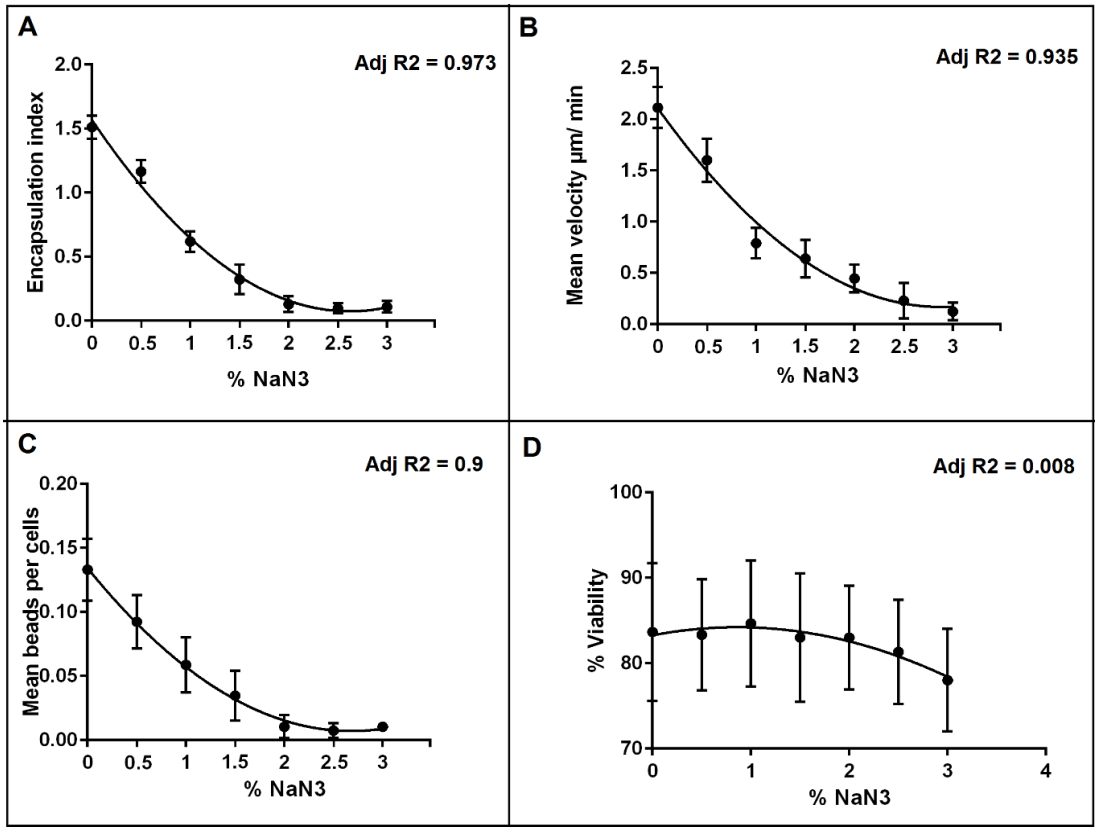


Figure 3.4. Dose-response relationships between the metabolic inhibitor NaN_3 and different endpoints of hemocyte health. *A) encapsulation index; B) mean velocity; C) phagocytosis and D) % viability, plotted points represent the SEM of 3 independent replicates.*

These results give confidence in the ability to accurately measure the determined immune-responses using the selected assays and cells, furthermore, observed effects are unlikely to be confounded by cytotoxicity of the selected pollutants in the ranges used. More extensive examination of the methodology of these assays and their application to the test chemicals will be described in detail in their respective chapters.

Chapter 4: *Encapsulation*

4.1. Introduction

Once a miracidium has successfully penetrated a snail it may rapidly be subjected to strong tissue reactions from the host which ultimately determine the outcome of the infection (Mohammed *et al.*, 2011b). The degree of this response is dependent on several factors including the developmental and nutritional state of the host and perhaps most importantly, genetic compatibility between parasite and snail (Santos *et al*., 2011). Also of varying, and in many cases undetermined, importance in influencing the outcome of the interaction are an innumerable assortment of environmental factors such as temperature, light, pH and the presence of xenobiotics; the latter of which is the subject of further investigate here (Santos *et al*., 2011).

The scope of this study was restricted to the very first stages at which the hosts ability to respond is evident and consequently also the first signs of any potential disruption by external factors.

Hemocytes migrate toward the sporocyst immediately upon recognition of a non-self-object and noticeable encapsulation will occur within 3 hours, followed by the phagocytosis of particles such as the epidermal plates after around 7.5 hours (Jourdane and Cheng, 1987; Mohammed *et al*., 2011). If the infection is prevented, the parasite will usually cease to develop at around 24 hours' post-penetration and death will occur within 72 hours (Mohammed *et al*., 2011).

While mechanisms such as the release of reactive oxygen species primarily determine the ultimate fate of the parasite, it is clear is that they must first be preceded by encapsulation. Interestingly, the encapsulation process occurs in either resistant or susceptible strains (Meuleman *et al*., 1987; Goodall *et al*., 2004). Some parasites may be destroyed by susceptible strains, equally some may develop in resistant strains, but it is the comparatively low rates at which these events occur that marks the difference.

Determining the exact role of encapsulation is complicated since some parasites in susceptible strains remain unencapsulated and are able to develop while others, which are equally viable, become totally surrounded by hemocytes (Ruelas *et al*., 2007). It appears that some parasites are able to develop regardless of encapsulation, but if destruction is to take place encapsulation must first occur.

In line with the establishment of various resistant and susceptible inbred stains of *B.glabrata* and the establishment of the Bge cell line, the development of *in vitro* techniques to manipulate the parasite-snail interaction has been of great importance in facilitating my understanding of the relationship between *B.glabrata* and *S.mansoni* (Yoshino and Coustau, 2011). The cell-mediated-cytotoxicity (CMC) assay first developed by Bayne *et al*., (1980) used *in vitro* transformed sporocysts to interact with collected hemocytes to investigate the nature of the killing mechanism. The basic principles of the assay, i.e. simulating the parasite-host reaction *in vitro*, have since been used for several purposes and I therefore applied it to study the effect of environmental pollutants.

As with several of my *in vitro* studies, this is the first instance where such work has been carried out with *B.glabrata-S.mansoni*, in the presence of chemical pollutants. In fact, I was unable to find evidence of *in vitro* encapsulation of parasites being used in any ecotoxicology study.

My aim was firstly to assess whether *in vitro* encapsulation assays could be adapted for use in ecotoxicological assays and secondly to determine the nature/degree of any relationship between encapsulation and xenobiotic exposure.

4.2. Materials and methods

The following section describes the methods specific to the preparation and conduction of the *in vitro* encapsulation assays.

4.2.1. Husbandry of test species

Snails and parasites were maintained and prepared as outlined in Chapter 2 (Section 2.1).

4.2.2. Fixing of sporocysts

Since the parasite life cycle was not available in-house I relied on eggs harvested from mouse livers collected at LSHTM to transform the parasites. Due to this, I was

limited to obtaining new eggs once every 6-8 weeks (in accordance with the parasite life cycle). For this reason, and due to the fact that I typically performed numerous assays over several days, I decided to fix sporocysts prior to use in the encapsulation assay. This was just one of several instances where decisions had to be based partly on practicality. This begin said, if using the correct type (and ratio) of fixatives, sporocysts which are fixed can induce much the same response as live parasites and may even provide advantages other than just practicality. Unlike internal features, including antigens, external features and their immunoreactivity are well preserved by fixatives (Lumsden *et al*., 1979). Fixing parasites also prevents protein turnover and so provides a more stable target (Bayne *et al*., 1986).

I used Karnovsky's fixative, which is the preferred choice for sporocysts destined for *in vitro* manipulation, rather than procedures such as electron microscopy. This solution is a mixture of 2.5% glutaraldehyde plus 2.5% formaldehyde in CBSS and makes use of the beneficial properties of both aldehyde fixatives (Renshaw, 2007; Nacif-Pimenta *et al*., 2012).

After transformation the sporocysts were first washed of the culture medium. To achieve this, parasites were gently extracted from the culture dishes and added to a 15ml falcon tube then mixed with 10ml CBSS. The tube was placed on ice to reduce further development and allow the sporocysts to naturally settle to the bottom of the tube over 15 minutes. The supernatant was then extracted and replaced with the fixative solution at room temperature. The parasite 'stock' can be used after 1 hour of fixing or potentially after considerably longer periods of time.

After fixing, the number of sporocysts was determined based on counting a representative sample under the microscope. The miracidia collection device described in Chapter 2 (Section 2.3.4) made this process much easier since there were no large pieces of debris which could be mistaken for parasites.

After the assay had been performed, the hemocytes/sporocysts were also fixed with the same solution prior to quantification, in order to halt the interaction but also to preserve it so that it could be quantified at a later date if necessary.

4.2.3. Development of chamber slides

While encapsulation responses can be viewed in a well-plate (using an inverted microscope), this is not ideal as the depth and small space mean visibility is difficult and objects may overlap. For this reason, samples were transferred to a microscope slide for quantification. The difficulty I encountered was that when interactions took place in well plates, as is typically the case, the addition of fixative caused many parasites/hemocytes to adhere strongly to the bottom of the plate which therefore could not be transferred to a slide.

The solution to this problem was to use a 'chamber slide' where the interaction takes place in a chamber which is attached to a microscope slide, with the surface of the slide forming the bottom of the chamber. The chamber can then be removed, resulting in samples settled on a microscope slide without needing to be transferred. While numerous commercial chamber slides exist, I was unable to find any which suited my particular needs. Commercially available chamber slides are almost exclusively aimed at cell culture monolayers and each slide consists of a chamber separated into multiple compartments to optimize space. This is suitable when working with a monolayer, as cells with grow within the chamber boundaries and can subsequently be viewed as separate/separated replicates on a single slide. Due to the nature of my assay, in order to ensure proper quantification each sample needed to be spread across the whole of a single slide and so I could not use multiple compartments on a single slide and was unable to find any existing designs which included only a single chamber. Another downside of these slides is their high cost which, when not able to perform multiple replicates on a single slide, was prohibitively high. For the reasons described above I developed a simple chamber slide using inexpensive materials to meet my exact requirements (Figure 4.1; A).

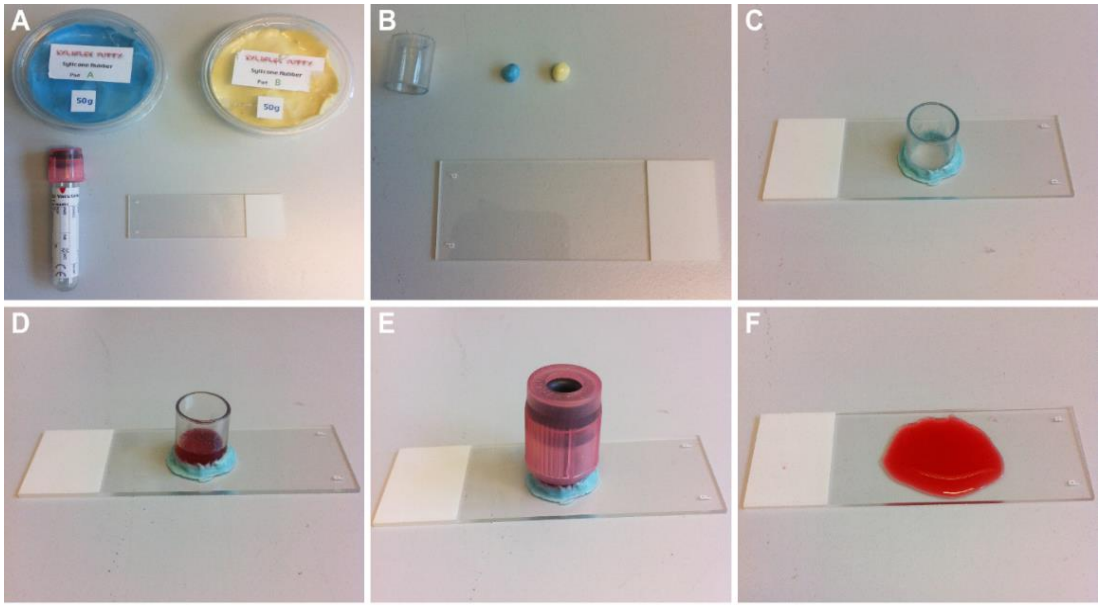


Figure 4.1. Development process for my purpose-built chamber-slide for use in the *in vitro* encapsulation assay. *A) The individual components used to construct the slides; silicone putty consisting of two parts, Vacutainer tube and glass microscope slide B) The individual components prepared for set-up; the Vacutainer tube cut to size and the appropriate amount of the two putty parts C) Chamber slide prepared for use, with the putty parts combined and hardened D) Chamber filled with hemolymph E) Vacutainer top added back on to the tube section to prevent desiccation/evaporation F) The resulting state of the hemolymph sample after removal of the tube chamber and putty; no residual putty left behind, the sample is now ready for application of a cover-slip.*

The basic principle of creating a chamber to place on the slide was extremely simple. For the chamber I used a siliconized Vacutainer tube since hemocytes do not stick to the sides of these tubes (Bezerra *et al*., 1999). The vacutainer was cut to a size able to accommodate 1 ml of liquid (Figure 4.1; B). Much more difficult was finding an appropriate method of keeping the chamber adhered to the slide so as to be water tight during the assay, while allowing the chamber to be removed for quantification. The majority of adhesives capable of sealing the chamber to the slide leave a residue which prevents the sample being spread over the slide and also typically contain toxic chemicals. I found a non-toxic silicone putty which was ideal for my purposes (Figure 4.1; A). The putty consists of two parts which when mixed together solidify in approximately 5 minutes (Figure 4.1; B). When applied around the base of the chamber the putty keeps a watertight seal which can be easily removed without leaving any residue (Figure 4.1; C-F). During the incubation stage of the assay the

vacutainer top was replaced onto the chamber to prevent desiccation (Figure 4.1; E). After the assay was complete the majority of supernatant could be removed, followed by the chamber, leaving the settled sample in a small volume of liquid which could then be spread by a coverslip for observation of a sporocyst monolayer (Figure 4.1; F).

To sterilize the chamber slides the individual solid parts were first soaked in 70% ethanol and allowed to dry under a cell-culture hood. The components were assembled under the hood and the slide was then subject to UV for 30 minutes.

4.2.4. Surface coating

The very first experiments into the *in vitro* interactions between sporocysts and hemocytes took place on an agar surface (Bayne *et al*., 1980). The purpose of this was to ensure a more 'natural' interaction. Hemocytes readily stick and spread to standard cell culture surfaces as well as to the parasite, coating plates in a non-adherent material such as 1% agarose ensures that hemocytes remain rounded unless in contact with the parasite, as would be the case *in vivo*.

I initially attempted to use the agar method but found certain aspects of the manipulation process to be awkward. Since agar is not transparent the interaction cannot be observed by a microscope *in situ* and sporocysts instead need to be carefully transfer to a microscope slide for observation (Bayne *et al*., 1980; Araque *et al*. 2003).

Boehmler *et al*., (1996) built on the original assay to allow a similarly natural interaction, but without the need for agar and thus allowing for observation to take place in the same vessel as the interaction. This was achieved by coating the plates with Poly-L-lysine which, at lower concentrations (0.1 mg/ml), prevents *B.glabrata* hemocytes from sticking to the culture surface. Interestingly this compound displays the opposite effect in cells from other species, where it is used to enhance adhesion and spreading (Boehmler *et al*., 1996).

In my final tests I chose to use the coating method described by Boehmler *et al*., (1996) and found that the cells responded in much the same manner they described. Other aspects of my encapsulation assay were considerably different to those

previously reported and required a reasonable degree of development in order to optimize them for my specific purposes.

4.2.5. Staining parasites

After fixing, the parasites were stained with FITC (Fluorescein isothiocyanate) since it can be extremely difficult to differentiate fully encapsulated sporocysts from cell aggregates which don't contain any parasites (Dikkeboom *et al*., 1988). Staining was adapted from the protocol used by Boswell and Bayne, (1986).

On the day of an assay, FITC was prepared at 5µg/ml in CBSS, added to the desired number of sporocysts and incubated in the dark for 25 minutes. After the incubation period a washing step was performed whereby the sporocysts were spun at 2000rpm for 5 minutes and the supernatant removed and replaced with an equal volume of FITC-free CBSS. This process was repeated approximately 3 times until the CBSS was free of visible stain and the tube was gently vortexed to separate the sporocysts.

4.2.6. Hemolymph and incubation

Hemolymph was collected as described in Chapter 2 (Section 2.2.2). 3 separate pools were made, each containing equal amounts of hemolymph from 8 snails and placed on ice. For each separate slide/chemical dose a sample of 200µl hemolymph was taken from each pool, mixed with an equal volume of 2x (double-strength) chemical in CBSS and added to the chamber slide (n=3 for each dose). The result was 400µl of 50% hemolymph and 50% CBSS/chemical in each chamber slide, with the correct chemical concentration achieved after accounting for the dilution by hemolymph. Stained sporocysts were added to the chamber slide at a ratio of 2 per µl of hemolymph, the chamber was then sealed and incubated at 27ºC for 4 hours. After the incubation period most of the liquid was removed leaving only approximately 50µl, along with the settled sporocysts and hemocytes. A cover-slip was placed over the sample and the slide then taken for counting under a fluorescence microscope.

4.2.7. Analysis; quantifying encapsulation and statistical analysis

The majority of the few studies which have investigated *in vitro* encapsulation in invertebrates typically quantify the process using a semi-quantitative 'encapsulation' or 'adhesion' index. Since it is not possible to quantify the exact percentage of a sporocyst that is encapsulated, an observer-defined value is assigned to each parasite. The values are based on either the approximate degree of encapsulation of the surface or the approximate number of cells attached (Table 4.1; Figure 4.2; Figure 4.3).

Table 4.1. Some examples of semi-quantitative scoring definitions of encapsulation index from various studies.

Reference	Index value	Definition of criteria for index value (Percentage coverage or number of cells attached)
(Kawasaki *et al*., 2013)	1	0
	2	20-40%
	3	>40-60%
	4	>60%
(Martins-Souza *et al*., 2011)	0	0
	1	1-10 cells
	2	11-50 cells
	3	>50 cells
(Castilllo and Yoshino, 2002)	1	0
	2	Up to 10 cells
	3	>10 cells, <50%
	4	>50%
(Bayne *et al*., 1986; Loker *et al*., 1989).	0	Single cells
	1	'point' contact
	2	'extensive contact'

In this study, the index used by Castilllo and Yoshino (2002) was adopted. All sporocysts on the slide were counted and assigned values of 0, 1, 2 or 3. The

individual values were tallied to give a total score which was then divided by the total number of sporocysts counted, to produce the final encapsulation index value.

$$\textbf{Assay encapsulation index value} = \frac{\textbf{Mean score of all sporocysts}}{\textbf{Total sporocysts counted}}$$

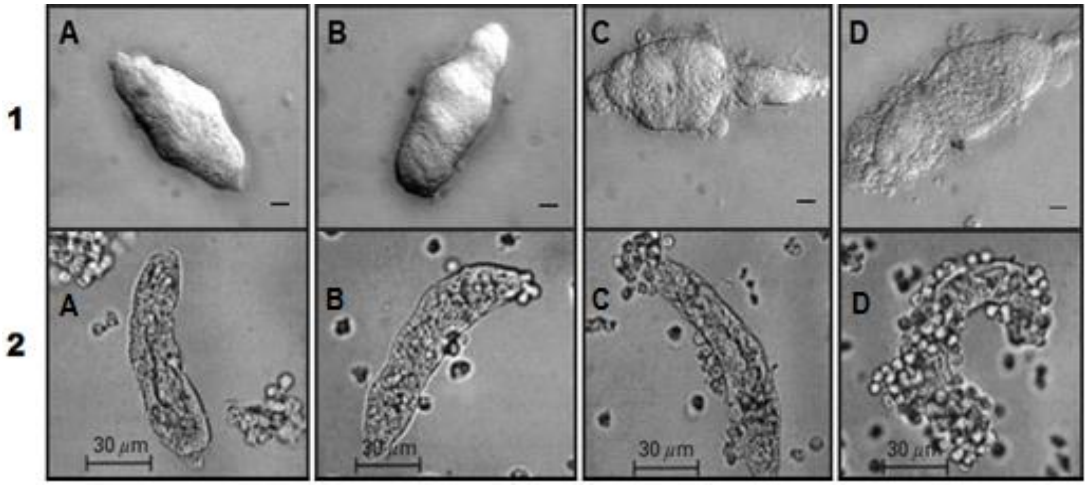


Figure 4.2. Photographic examples of sporocyst encapsulation index from two different studies. *Row 1, A-D; encapsulation index values 0-3 according to Martins-Souza et al., (2011). Row 2, A-D; encapsulation index values 1-4 according to Castilllo and Yoshino (2002).*

Statistical analysis was performed in GraphPad Prism 6.0 for Windows (GraphPad Software Inc., La Jolla, CA) and the main principles adhere to those discussed in Chapter 2. Statistical tests were chosen with the help of the OECD Document on Current Approaches to Statistical Analysis of Ecotoxicity Data (OECD, 2009). For the purpose of analysis 'encapsulation index' was considered as a measurement value. Despite sporocysts being given a subjective rating McDonald (2014) explains that this type of data, where objective measurement using instruments is not possible, can still be classed as a measurement even though the measurement is performed by the observer, since values still fall on an interval scale.

The relationship between dose (X) and encapsulation index score (Y) was modelled by first, second or third order polynomial regression models. All values for Y were entered as three independent observations (n =3) and values were not averaged prior to analysis, nor were they transformed. X values were log transformed.

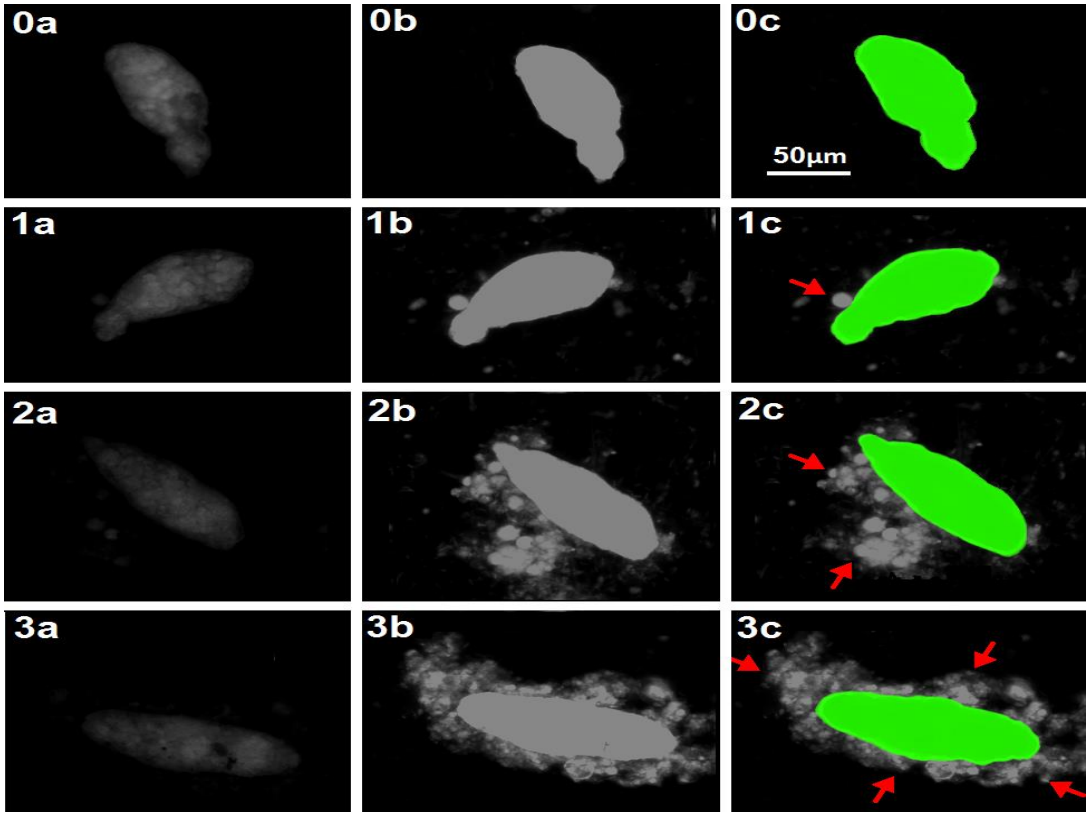


Figure 4.3. Example of fluorescence microscopy used in the scoring index of my encapsulation assays. *A) Example of how fluorescent sporocysts can be identified even when encapsulating B) Shows that adjustment of exposure and contrast allows observation of a 2D image of the sporocyst and the attached cells C) Shows the sporocysts after being masked to better distinguish the parasite from the surrounding cells. Arrows indicate attached hemocytes.*

4.3. Results

When compared to images from Martins-Souza, *et al*., (2011) and Castilllo and Yoshino, (2002) (Figure 4.2; 1 and 2), my interactions appear morphologically very similar, giving confidence in the counting procedure used as it was the same as they describe and also suggests that interactions were essentially as would be expected under 'normal' circumstances.

All chemicals were best described in their relationship with the encapsulation index by a nonlinear dose-response curve. The relationship between encapsulation index and dose for BPA and the effluent extract was best modelled by a second order (quadratic) polynomial ($y=a+bx+cx^2$) curve whereas E2 was best modelled by a third order (cubic) polynomial ($Y = a + bx + cx^2 + dx^3$). The dose-response relationship

for DDE was the most simplistic, being best described by a first order (straight line) polynomial model ($y = b0 + b1x$) (Figure 4.4; D).

All outputs for the regression models were free of error messages indicating that the fundamental parameters were appropriate i.e. the fit was not ambiguous, there were sufficient data points and confidence intervals were not excessively wide.

For all models, the D'Agostino-Pearson omnibus K2 test was not significant (p = >0.05) suggesting that the residuals were normally distributed and thus fit the requirements for regression.

In order to account for the complexity of higher-order polynomial models and the resulting potential for inflating the R^2 value, I chose to only report the adjusted R^2 as it provides a more realistic interpretation (section 3.3.3). For E2 I obtained an adjusted R^2 value of 0.225 suggesting that the majority of the variance in encapsulation index (77.5%) is not explained by E2 dose using this model. The models for the remaining chemicals displayed higher adjusted R^2 values, with dose typically explaining around 50% of the variance in encapsulation index with values of 0.5276, 0.4584 and 0.6017 for BPA, effluent and DDE respectively.

Since I collected multiple independent values for Y at each value of X I was able to run a replicates test to determine whether the average distance of the points from the curve was significantly different from the among-replicate scatter i.e. whether the curve is close enough to the mean of the replicates. This test is a good indicator of the suitability of a model and in each case I obtained a test significance level of >0.05 indicating that the chosen models are likely to be adequate.

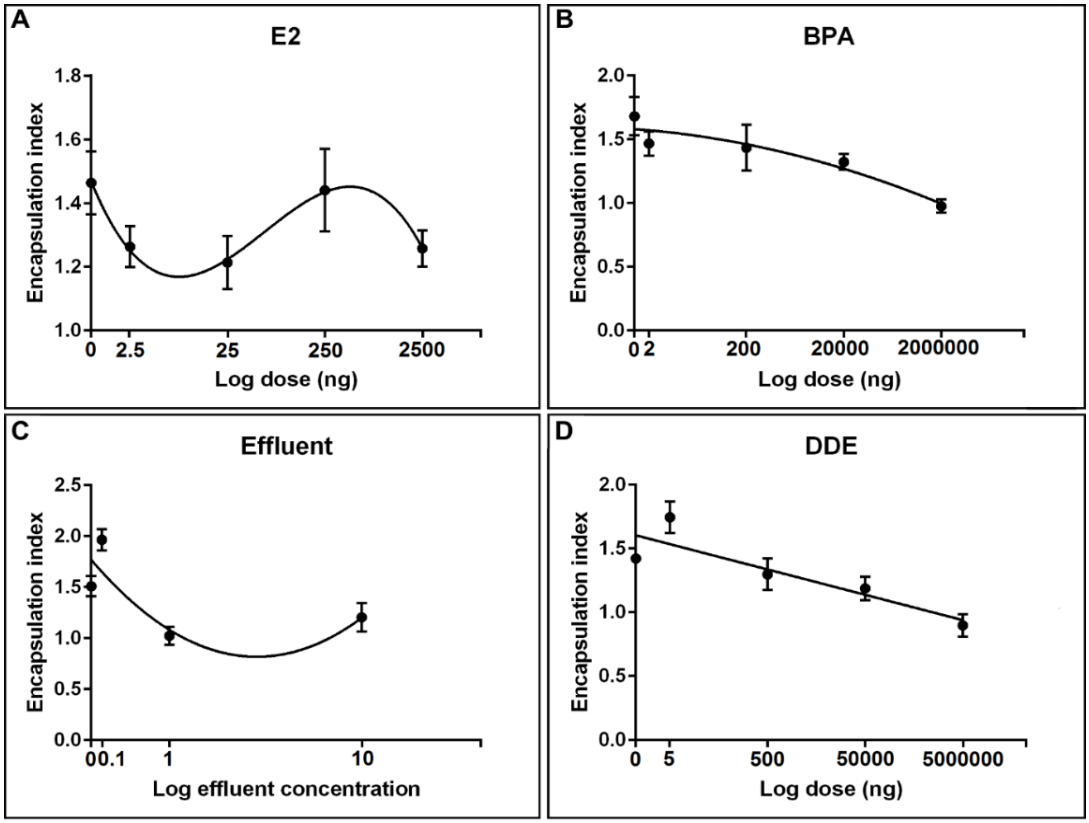


Figure 4.4. Regression relationships between log dose (X axis) of test chemicals against encapsulation response toward *S. mansoni* sporocysts by *B. glabrata* hemocytes exposed *in vitro* (Y axis). *Hemocyte response plotted as mean encapsulation index value ± SEM of 3 independent replicates. A) Hemocyte encapsulation response to E2 exposure B) Hemocyte encapsulation response to BPA exposure C) Hemocyte encapsulation response to Effluent exposure D) Hemocyte encapsulation response to DDE exposure.*

As described in Chapter 3, Section 3.3.7, I decided to further explore the data in a more 'general' manor by using Dunnett's test for multiple comparisons to look for any significant differences between the encapsulation index at an individual dose when compared to the respective control (0ng) for that chemical.

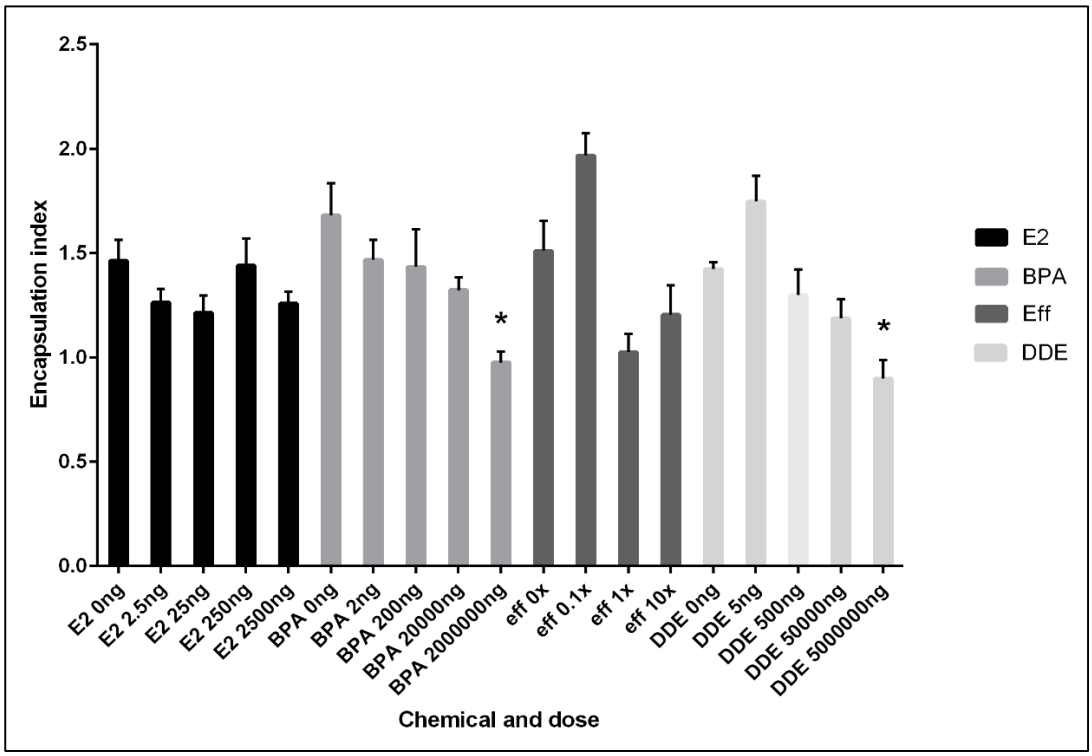


Figure 4.5. Dunnett's multiple comparisons between mean value of hemocyte encapsulation index (X) at each test chemical and dose (Y). *Hemocyte response plotted as mean encapsulation index value ± SEM of 3 independent replicates for all doses of each chemical (Y). Response to doses of the same test chemical in different shades. Asterisks indicate doses which were significantly different to their respective control (p = < 0.05) according to Dunnett's test for multiple comparisons.*

The results from the multiple comparisons test showed that only the top doses of BPA (2mg/L) and DDE (5mg/L) were significantly different from their controls (Figure 4.5).

Tukeys HSD multiple comparisons test was also performed to compared all chemicals and doses with each other. Numerous significant differences between combinations of different doses/chemicals were found, with 0.1x effluent appearing in the largest number of significant combinations. Within chemicals, aside from the differences shown by Dunnett's test, a significant difference was found between 0.1x and both 1x and 10x for effluent and between 5ng and 5mg for DDE.

4.4. Discussion

The constructed chamber slides proved to be a simple and effective means of studying the interaction given the limitations of the available equipment. These chamber slides could likely be applied to various different *in vitro* cell-based assays beyond those described here, especially in situations where cost is a significant factor (individual chamber slides typically retail at between £10-£15 and are usually only available in packs of a minimum size of 16).

Only a small number of *in vitro* hemocyte encapsulation assays are reported in the literature for any species and an even smaller number concerning *B.glabrata*. I was able to qualitatively compare my assay to those studies which included images of the *in vitro* encapsulation process which they had observed (Figure 4.2) and this gave me confidence in the validity/normality of the interactions between sporocyst and hemocytes in my study.

To my knowledge there exist no other studies which have investigated *in vitro* encapsulation by invertebrate hemocytes in response to environmental contaminants. Since this area has not been investigated in a detailed fashion, I am restricted in the generalizations I can make and, unlike the basic hemocyte-sporocyst interaction, there is little against which I can compare and contrast findings in relation to chemical dose-response. Despite this, there do exist a number of studies on the effects of some of the study chemicals (primarily BPA and E2) on *in vitro* immune parameters in molluscs (mussels), the majority of which come from the work of Canesi *et al*. While their work does not address the encapsulation response, it is known that certain key intracellular immune-related and/or regulatory processes can be responsible for directly or indirectly controlling several different functions. For example, Canesi *et al*., (2004) showed that E2 was able to rapidly effect different immune parameters in mussel hemocytes through modulation of Mitogen-activated protein kinases (MAPK) *via* the activation of kinase cascades. Interestingly, but perhaps not surprisingly since such functions are often well conserved, these same signalling pathways are believed to be important in regulating cell adherence and spreading in *B.glabrata*, which are two of the fundamental stages leading to encapsulation (Humphries *et al*., 2001). From this we can speculate as to one possible mechanism by which E2 may influence encapsulation, however based on

the data obtained for this chemical it would be impossible to make any conclusions without performing more studies, particularly at the molecular level.

Nonlinear dose-response patterns are frequently seen in toxicology and as a result polynomial models are increasingly described in the literature (Davidson *et al*., 2006). Models which once may have been considered 'too complex' are now often seen as being more realistic when describing the relationship between certain compounds and biological functions in a range of species including molluscs (Canesi *et al*., 2007b; Forbes *et al*., 2007; OECD, 2009).

Non-monotonic relationships, such as that which I found for E2, typically suggest that numerous different mechanisms are involved in the response and this may be the case for encapsulation which is thought to be under the control of several. Canesi *et al*., (2004) found that BPA also caused significant disruption of immune parameters but did not elicit its response *via* exactly the same mechanisms as E2. In a later study involving BPA Canesi *et al*., (2007) performed dose-response analysis against several immune parameters of mussel hemocytes and in several cases the relationship was found to be non-monotonic in nature.

While there is insufficient data in the literature to make any comparisons regarding the possible mechanisms behind the *in vitro* effects of DDE or effluent extract exposure on mollusc hemocyte immune parameters, the results for these chemicals do offer some potential, yet fairly broad, explanations as to the nature of the relationship.

DDE and the effluent extract appear to display hormesis-like trends, characterised by low-dose stimulation and high-dose suppression. This phenomenon has been reported in a wide range of animal and even plant species and is also often associated with immune function dose-response relationships (Calabrese, 2008). Tukey's HSD test was a useful means of confirming this, as for both chemicals the lowest (non-control) dose was associated with a significantly higher encapsulation score than the highest dose. In both cases the lowest dose can be seen to be higher than the control (Figure 4.5), although differences were not significant. The underlying cause of this type of relationship is still unclear but it is sometimes suggested that stimulation occurs as an overcompensation to a low, but non-toxic, dose which disrupts

homeostasis, whereas high doses suppress activity, possibly because they simply become acutely toxic (Calabrese, 2008).

4.5: Conclusion

I was able to successfully perform *in vitro* encapsulation interactions which were comparable to those reported in previous studies and was able to do so using a low-cost, yet effective chamber-slide based on easily acquirable items. A simple technique such as this may appeal to those who wish to perform a small number of chamber-slide assays and do not wish to purchase in bulk or those who wish to perform numerous assays and cannot afford to spend hundreds, or even thousands, of pounds on disposable commercial chamber slides.

I have shown that it is feasible to perform *in vitro* encapsulation assays under the exposure of selected chemicals and to perform quantifiable dose-response assays. To my knowledge *in vitro* encapsulation has not previously been used as a means of assessing pollutant-induced impairment of immunity and so primarily I consider this work as a proof-of-principle first and foremost.

I was able to show that certain environmental pollutants do appear to exert a modest effect on *in vitro* sporocyst encapsulation, but that the relationship is relatively complex, as is often the case when dealing with endocrine disrupting chemicals. However, the aim was not to intentionally achieve a dramatic and stark response by including doses which entirely disrupt the process. The doses used in this study maintain a degree of environmental relevance (based on water concentration alone), although it is likely that concentrations of some chemicals in hemolymph, *in vivo*, could be significantly higher due to bioconcentration.

Pinpointing the exact mechanisms responsible for the observed effects was beyond the scope of this study, but I suggest that this assay could be performed with more specific end-points in mind to answer such questions, perhaps at a molecular level.

Chapter 5: *Phagocytosis*

5.1. Introduction

Perhaps the most important function of hemocytes and one of the key parameters of the non-specific immune response in all animals, is phagocytosis (Fournier *et al*., 2000; Gust *et al*., 2013). Upon encountering a potential pathogen, hemocytes attach to the object and attempt to internalize it, effectively incorporating the object into the hemocyte itself, at which point it is subject to intracellular destruction by reactive oxygen species (Lopez-Cortes *et al*., 1999; Gust *et al*., 2013).
In the case of larger objects which a single cell cannot internalize, hemocytes still attempt phagocytosis but this typically results in many cells spread over the surface of the object until it is entirely surrounded. This process is known as encapsulation and the result is similar to granuloma formation in vertebrates (Cheng, 1981; Galloway and Depledge, 2001). Together the mechanistically identical processes of phagocytosis and encapsulation represent the primary response of the molluscan immune system to foreign objects. For this reason, phagocytosis has become an important and well established biomarker of immune function in molluscs and other species.
Phagocytosis has been measured in bivalve and gastropod mollusc hemocytes using a variety of different methods that typically involve enumeration of ingested particles (Blaise *et al*., 2002).

Standard bright-field microscopy has been used to study phagocytosis for over 100 years; a process that was first observed in hemocytes (Cooper *et al*., 2006). The main draw-back of this technique is the difficulty in distinguishing between particles that have been internalized and those that are simply attached to the cell surface; something which is fundamental to accurately assessing phagocytosis (Champion, 2007). Fluorescence microscopy helps to overcome this problem based on the properties of different staining techniques and the ability to quench and exclude external particles (Lopez-Cortes *et al*., 1999).

Microscopy can provide accurate and detailed information regarding individual cell morphology and phagocytosis but is labour intensive and time consuming, especially given the large sample sizes often required to overcome the variability between individual hemocytes (Oehlmann and Schulte-Oehlmann, 2002; Goedken *et al*., 2004;). Furthermore, phagocytosis measurements made using standard microscopy

may be prone to experimental bias due to the subjectivity of user scoring (Payés *et al*., 2012).

In the late 1980's flow cytometry enabled substantial increases in the speed and sample size available to phagocytosis assays using mollusc hemocytes (Fisher and Ford, 1988; Alvarez *et al*., 1989; Ashton-Alcox and Ford, 1998). Despite these advantages, flow cytometry is limited by the fact that cells are not directly visualized and quantification is based solely on fluorescence intensity. Therefore, some authors consider flow cytometry to be a 'semi-quantitative' method (Ploppa *et al*., 2012).

Recent advances in camera, optics and computing technology have made it possible to produce commercially available imaging flow cytometers (IFC). Imaging flow cytometers combine the strengths of microscopy (including spatial, morphological and fluorescence information) with the high-throughput nature of flow cytometry and are therefore perfectly suited to perform accurate and statistically robust phagocytosis assays (Filby and Davies, 2012). As well as the increased amount of data which is taken from each cell, most imaging flow cytometers come with advanced software which can greatly improve the accuracy of gating since each individual point on a scatter graph can be directly related to the corresponding cell image for subsequent visual confirmation (Zuba-Surma *et al*., 2007).

In this study I used hemocytes derived from two species of gastropod to developed novel quantitative methods that take advantage of IFC technology, with the aim of modernizing hemocyte phagocytosis assays. To the best of my knowledge this is the first study in which imaging flow cytometry has been applied to mollusc hemocytes and the first attempt to investigate phagocytosis, in any manner, in *Marisa cornuarietis.* Therefore, the data presented here provides new knowledge of phagocytosis in mollusc hemocytes and represents a technical advancement in the study of mollusc immune responses following exposure to chemical pollutants.

5.2. Materials and methods

The following section describes the methods specific to the preparation and conduction of the various *in vitro* phagocytosis assays.

5.2.1. Experimental species and husbandry

Biomphalaria glabrata (BBO2 strain) and *Marisa cornuarietis* were maintained under laboratory conditions as described in Chapter 2 (Section 2.1 and 2.4.5). For each different assay condition (i.e. treatment or dose, within the same experiment) cells were derived from the same pool. Independent experimental replicates were performed on different days using different pools of hemocytes.

5.2.2. Antigens

Several different objects were used as antigens to promote phagocytosis in the different studies. Each antigen had distinct advantages and disadvantages compared to the others. The use of different antigens provided greater scope to investigate hemocyte responses and also helped to determine which antigen was most suited for a particular assay/species. To illustrate, during preliminary tests optimal outcomes could not be achieved when exposing *B.glabrata* to yeast cells. In contrast, *M. cornuarietis* displayed excellent responses to yeast but not latex beads, to which *B.glabrata* responded well.

5.2.2.1. Latex beads

Latex beads are a commonly used antigen in phagocytosis studies on many species as they provide a relatively uniform size and high degree of fluorescence (Lehmann *et al*., 2000). I used 10µl of a 1/10 stock of 1.0µm green amine-modified latex beads (Sigma, L2778). The latex beads required this significant dilution due to their extremely large numbers, which made enumeration difficult and resulted in excessive amounts of 'free' beads in the sample. Moreover, high numbers of beads resulted in excessive rates of phagocytosis which obscured the finer nature and quantification of the process (due to large numbers of beads inside the cells).

5.2.2.2. Bioparticles

Recently a new dye (pHrodo™), has been developed by LifeTechnologies which displays increasing fluorescence with decreasing pH (Simons, 2011). Since the inside of phagosomes is typically much more acidic than the surrounding environment internalized objects that are coated in the dye (*E.coli* bacteria in the case of Bioparticles) show increased fluorescence over non-internalized objects. Thus, the degree of phagocytosis can be calculated based on fluorescence intensity against a control, without the need for extra steps to account for the exclusion of non-internalized objects, as is normally the case.

The dilution used for Bioparticles was 1ml of a stock solution of 1mg per ml CBSS per $1x10^6$ cells. The Bioparticles used were green in fluorescence emission.

5.2.2.3. Yeast

Yeast was labelled according to a protocol adapted from Morris *et al*., (2003). A solution of 13.5mg/ml live *Saccharomyces cerevisiae* (dried active yeast, Allinson) was made in distilled water and was heat-killed by boiling for 30 minutes in an Eppendorf tube. Next a carbonate-bicarbonate solution was made by combining 4ml of 0.2M sodium carbonate with 46ml of 0.2M sodium bicarbonate. The solution was then made up to 200ml with distilled water and the final pH was adjusted to 9.2. The yeast solution was then centrifuged at 300 x g for 5 minutes, the supernatant was poured off and the pellet re-suspended in equal volume of carbonate-bicarbonate solution with 500µg/ml FITC. The yeast solution was left on a shaker overnight. The following day the solution was centrifuged for 5 minutes at 300 x g and the supernatant replaced with Chernin's buffered salt solution (CBSS PH 7.4; Chernin, 1963). This step was repeated until the supernatant appeared to contain no stain (approx. 5 times). Prior to use in an assay, the yeast solution was vortexed for 1 minute and then passed through a 40µm filter to remove any large clumps. 40µl was added to the hemocyte solution in each case.

5.2.3. Exposing hemocytes to antigens; background

The study of phagocytosis in *B.glabrata* has traditionally involved allowing cells to spread on an artificial surface, after which they are exposed to the chosen antigen. One advantage of this method is that it allows cells to be washed free of any unwanted material and/or the addition of CBSS, while the adherent cells (also the phagocytosing cells) remain attached (Fryer and Adema, 1993). This method of separating cells from hemolymph is also advantageous due to the high mortality and clumping associated with centrifugation of hemocytes. The downside of this method is that it requires cells to be assessed whilst still spread and therefore they cannot be used for analysis that requires a cell suspension e.g. flow cytometry.

In recent years increasing numbers of studies on phagocytosis in molluscan hemocytes have utilized flow cytometry, however, I was only able to find one such study concerning *Biomphalaria* and none concerning *Marisa* (Bakry *et al*., 2012).

5.2.3.1. Exposing hemocytes to antigens; my method

To expose hemocytes to antigens, while also allowing them to be suitable for use in flow cytometry, I developed a 'hybrid' approach whereby I was able to combine the advantages of exposing to spread cells with the more advanced assaying equipment (flow cytometry) available only for use with a suspension sample.

400µl of pooled hemolymph was added to individual wells of a 24-well plate and the cells were allowed to adhere for 30 mins at 27^{o}C. After 30 mins the supernatant (including non-attached cells) was removed and replaced with 400µl of test solution in CBSS and incubated for a further 30 mins at 27^{o}C. After initial incubation with the test chemical, the antigens were added and the plates were incubated at 27^{o}C on a gentle shaker for 2 hours. The supernatant was then aspirated and replaced with 200µl of α-CE. After 10 minutes in α-CE cells were transferred to an Eppendorf and kept on ice until use in the ImageStream.

5.2.4. Imaging flow cytometery

Imaging flow cytometry (IFC) was performed using the ImageStreamX system (Amnis Inc., Seattle, Washington). The 40x objective was used to acquire images in each experiment.

5.2.4.1. IFC; Pre-acquisition

ImageStream settings are controlled using the INSPIRE software interface. The size of objects recorded is determined by user defined classifiers on an arbitrary scale. Prior to running the sample, classifiers can be set to exclude debris such as large cell clumps which have failed to separate, or any other debris not of interest. For each different cell/antigen type I adjusted the classifiers to achieve optimum collection of single cells. Laser power was also adjusted according to the antigen used. As some antigens have greater fluorescence intensity than others, the laser power was adjusted to prevent over- or under-exposure of the sample.

5.2.4.2. IFC; Post-acquisition

Unlike standard flow-cytometry, IFC allows data to be significantly refined post acquisition. Regardless of how well classifiers and/or focus and laser power are set, all data sets will inevitably contain recordings for undesirable objects. Some objects can be easily excluded due to their large or small size (through a process called 'gating') but IFC enhances our ability to exclude objects which may be of similar morphology to cells since we can visualize them and make decisions in a manner otherwise impossible. Cell image data collected from the ImageStream was analysed using IDEAS 6.0. A histogram was produced based on gradient root-mean-square (RMS) and a region drawn based on visual inspection to define cells which were in acceptable focus (Figure 5.1). Based on the focused population, a scatter graph of area vs aspect ratio was produced and a region drawn to define single cells while excluding clumps, non-cellular debris and free (i.e. non-internalized) antigens (Figure 5.2).

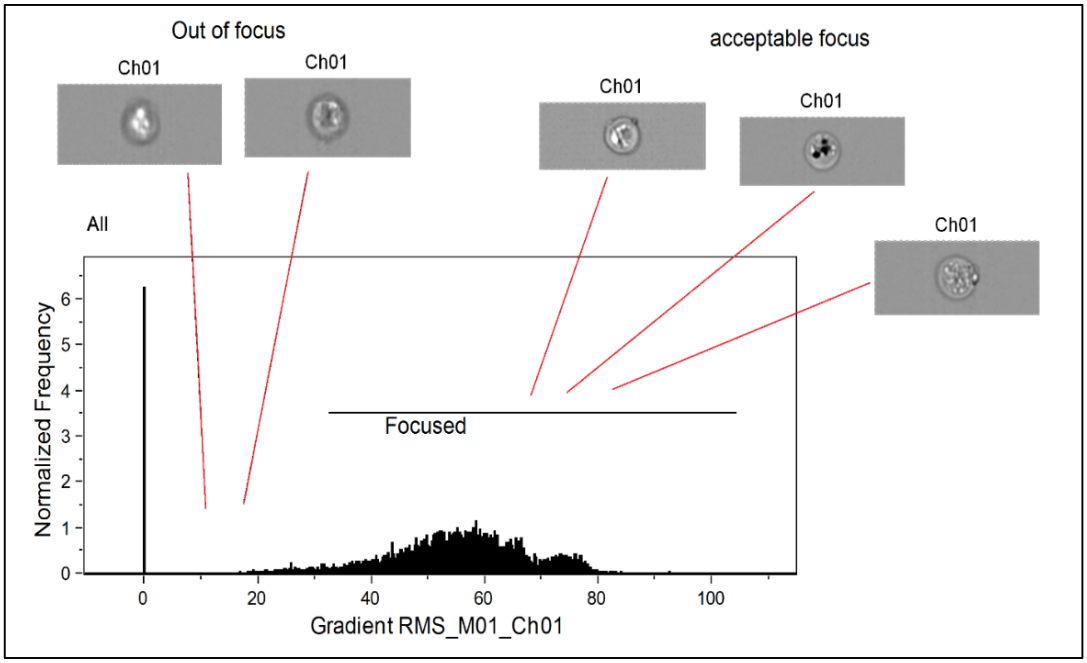


Figure 5.1. Identifying properly focused hemocytes using a frequency histogram generated with ImageStream gradient Root Mean Squared (RMS) data. *Hemocyte images were recorded on the brightfield channel (Ch01). 'Focused' line represents a region of cells which are in acceptable focus according to user-inspection of images; only this population will be used for further analysis. Example images are of hemocytes from focused and non-focused populations*

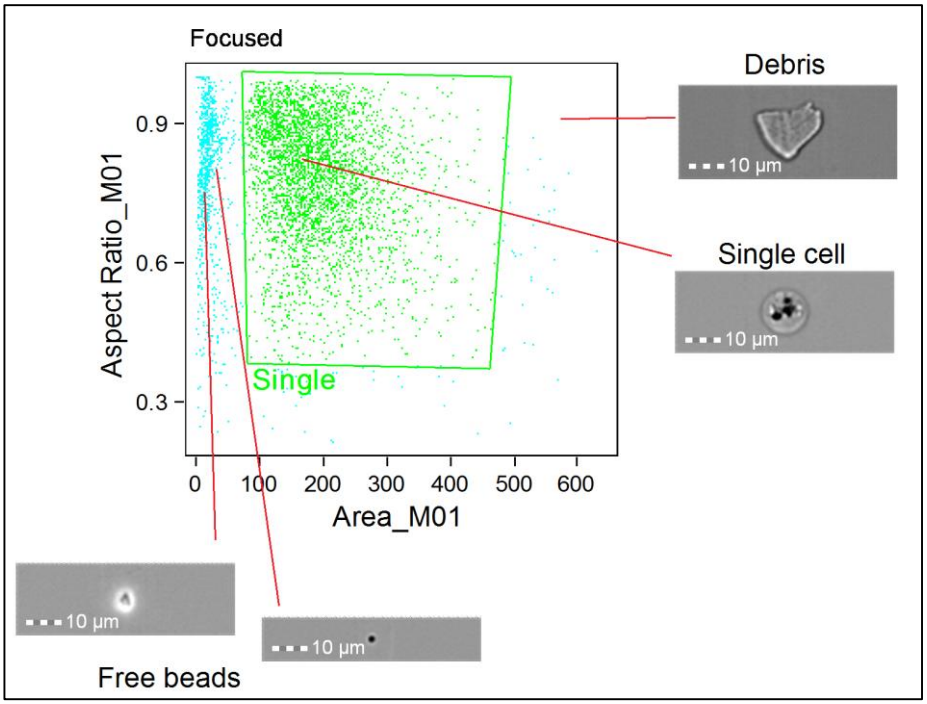


Figure 5.2. Identifying single cells using a scatter-plot based on ImageStream aspect ratio and area data from the 'focused' hemocyte population. *Region containing single cells are gated based on visual inspection of the brightfield images, example image of single hemocyte within the gated population and of non-cellular debris and free beads which have been excluded*.

5.2.5. IFC; masking

The fluorescence of beads is difficult to quench using trypan blue, due to their extreme intensity. While the majority of non-internalized beads are removed by gating (Figure 5.2) some beads are bound to the cell surface or come through simultaneously in the flow stream with the cell. In these cases a false-positive for phagocytosis may be recorded (Lehmann *et al*., 1998). I used IDEAS 6.0 to develop a masking strategy to help distinguish between bead-associated images representing phagocytosis and those which did not.

A histogram for channel 2 (green) fluorescence intensity was created based on the focused and single cell populations and a region was created for images which were positive for beads according to visual inspection (figure 5.3).

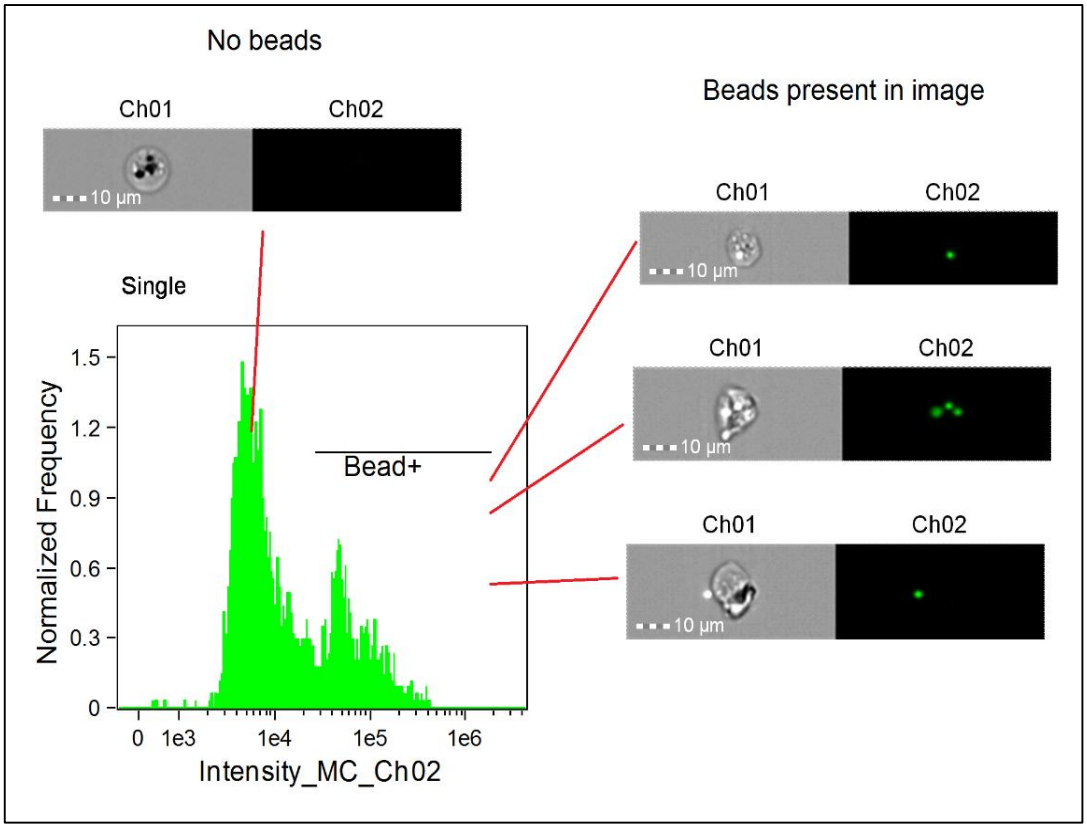


Figure 5.3. Identifying hemocyte images containing a fluorescent latex bead using a frequency histogram generated with fluorescent intensity data, based on the 'single cell' population. *Hemocyte images recorded on channel 1 (Ch01) and channel 2 (Ch02). Bead+ line represents a region of cell images which contain one or more beads according to user-inspection of images. Representative images from each population are included showing the brightfield channel with the cell and the fluorescent channel with or without a bead.*

To exclude cells in which the beads were not internalized, a morphology mask corresponding to the inside of the cell was created based on the bead positive population. To the brightfield image (Channel 1), an object mask was applied which is a general mask for any objects in the image, pixels were then eroded from this to restrict the mask to inside the cell and then dilated to fit the exact cell boundary (Figure 5.4). The effectiveness of the mask was tested against a 'truth population' of cells visually confirmed to contain internalized beads and those which contained non-internalized beads. To identify beads a spot mask was added on Channel 2 with a cell to background ratio of 35.75 and a radius of 1.

Figure 5.4. Example of the process of developing a custom mask in order to restrict the analysis to the cell image only and exclude external objects. *The first picture shows a composite of the brightfield and channel 2 images of a hemocyte with an external bead and no mask applied. The second image is the same as the first but with the application of an object mask to identify any items within the image. The final image of the same cell/bead shows the ability of the custom mask I developed to exclude external beads.*

After determining the bead positive population (Figure 5.3) based on Channel 2 intensity the custom mask was applied to this Bead+ population to determine the true extent of internalized beads from the cell images that are positive for beads, but in which beads are not necessarily internalized (Figure 5.5).

The mask was also able to successfully categorize images which contained both an internalized bead as well as a non-internalized bead (Figure 5.6).

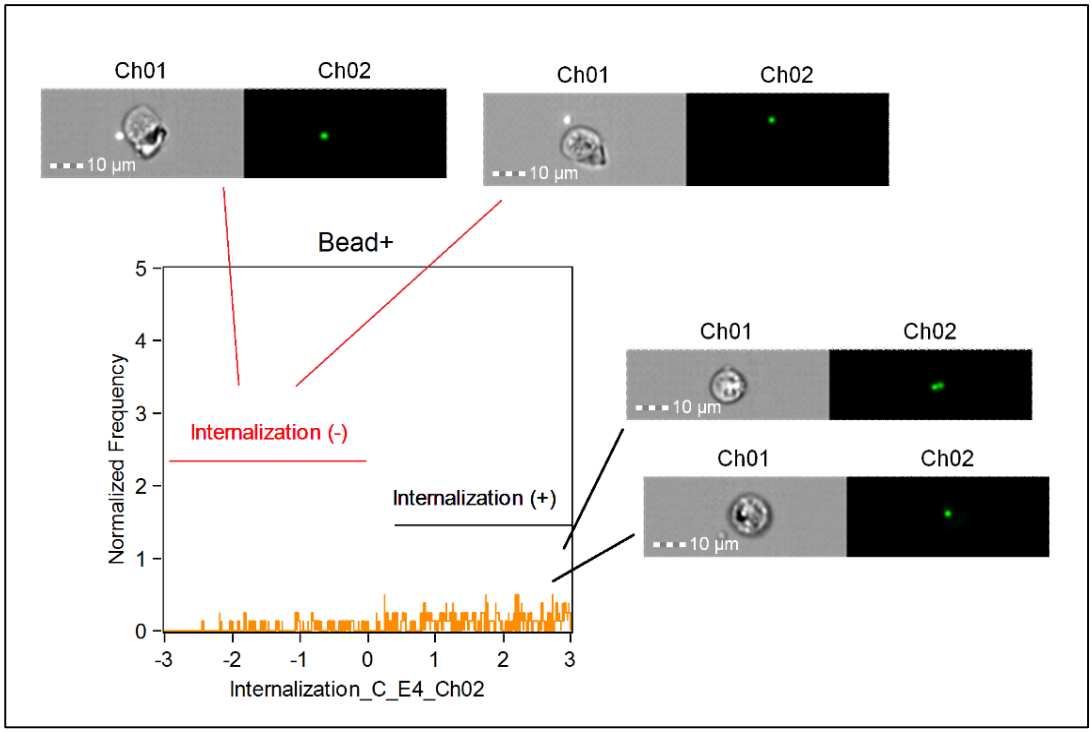


Figure 5.5. Frequency histogram based on the bead+ population gated into regions of images containing internalization positive (+) and negative (-) cells, according to the custom mask. *Hemocyte images recorded on channel 1 (Ch01) and channel 2 (Ch02). Internalization (+) line represents a region of cells which contain one or more beads according to the custom mask. Internalization (-) line represents a region of cells with no beads inside the cell, although they may be in the same image they have been excluded by the mask.*

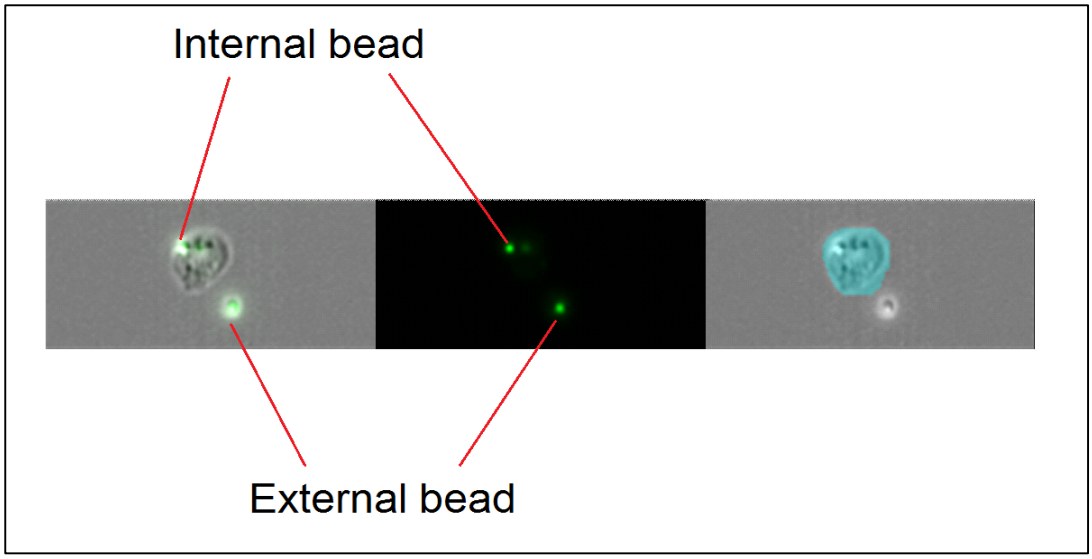


Figure 5.6. Example of a situation in which the custom mask correctly identifies an internalized bead while excluding a non-internalized one. *The image on the left is a composite of brightfield and channel 2. The central image is the same but shows channel 2 only, which enhances the contrast of the beads. The image on the right is again the same but with the application of the custom mask which includes the internalized bead but not the external one.*

To quantify the number of internalized beads a spot count feature based on the internalization positive population was created using the morphology/spot count mask. This function counts objects with high Channel 2 fluorescence which fall within the mask and thus gives us information on how many beads are inside each internalization positive cell (Figure 5.7). We expect the spot count histogram to show no data for '0 beads' since the counting was only performed on the cell population that had internalized beads. Should any images fall into this category then the mask would need refining since we would expect all cells counted to contain at least one bead.

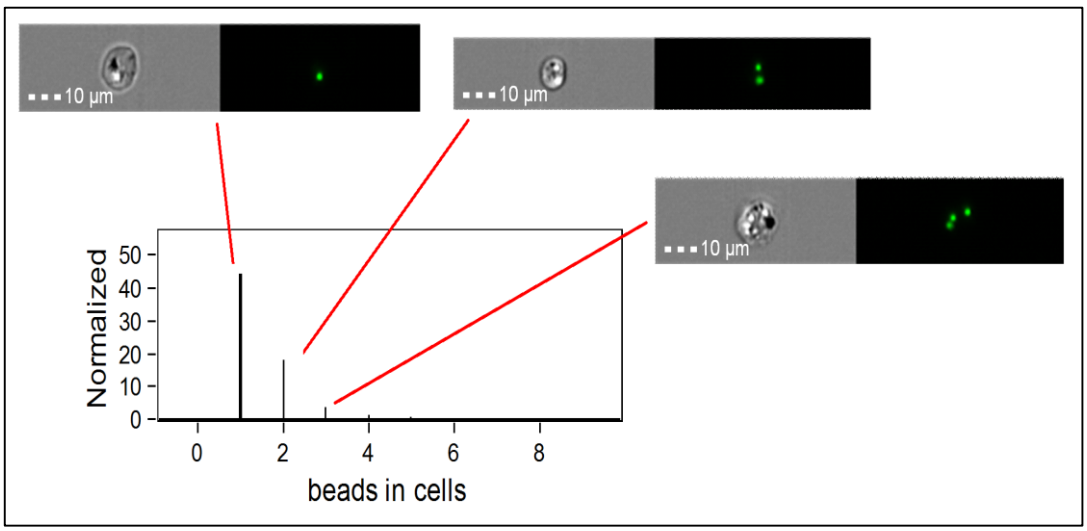


Figure 5.7. Frequency histogram based on the internalization positive population showing the quantity of hemocytes which contain specific numbers of beads. *Images show examples of cells which contained different numbers of beads.*

In order to validate the masking method, I performed a quantitative evaluation of the effectiveness of the mask by manually (visually) scoring each cell (>500) from an automated output.

5.2.6. *in vitro* phagocytosis assays

This section outlines the various assays that were performed to assess *in vitro* phagocytosis. Unless stated otherwise, the preparation for each of the following assays was as described in Section 5.2.

5.2.6.1. *B.glabrata*

The following sub-sections describe the preparation of the *in vitro* phagocytosis assays performed using hemocytes from the primary test species; *B.glabrata*.

5.2.6.1.1. Variation in hemocyte phagocytosis rates

Since hemocytes are known to be highly variable between individual animals and are thus often pooled, I tested the variation in phagocytosis response between technical replicates (intra-pool) and biological replicates (inter-pool). These cells were not treated with any xenobiotic or inhibitory chemicals.

5.2.6.1.2. *in vitro* exposure of hemocytes to study chemicals

Cells were prepared as a monolayer and were exposed to the same chemicals at the same concentrations as previously described. Latex beads were added as antigens. 3000 cells from 4 independent replicates (pools) were recorded for each dose. Classifiers were set at: 75-300, 488nm laser excitation: 20, 785nm laser excitation: 1.

5.2.6.1.3. Bioparticle; *in vitro* phagocytosis assay

Cells were prepared as a monolayer. 10ug/ml cytochalasin B in CBSS was used as a negative control since it has been shown to be a potent inhibitor of phagocytosis in mollusc hemocytes (Allam *et al*., 2002). All other parameters were the same as those used for the chemical exposure study.

5.2.6.1.4. MTT; *in vitro* phagocytosis assay

In addition to flow cytometry I developed a different method of assessing phagocytosis in order to give a broader approach but also to potentially present a much more simplistic and cost-effective method.

MTT (a tetrazolium salt) is reduced by cellular metabolic activity producing crystals of formazan (chestnut brown-bluish colour) and thus the colour intensity is proportional to the number of cells and the degree of their metabolic activity (Bezerra *et al.*, 1999). The MTT assay has routinely been used to assess viability in mammalian cells for years and can also be used to assess increased cellular activity above normal levels which occurs during high-energy processes like phagocytosis. While comparatively much less common, the MTT assay has also been successfully used for the same purposes in invertebrate cells, again demonstrating the remarkable degree of evolutionary conservation among processes. Bezerra *et al*., (1999) developed a protocol to compare rates of phagocytosis between human granulocytes and *B. glabrata* hemocytes.

In a similar manner to the encapsulation assays, the protocol described by Bezzera *et al.,* (1999) was adapted to incorporate exposure of hemocytes to test chemicals. This study, to my knowledge, represents the first instance of the MTT assay being used to study the effects of pollutant chemicals on phagocytosis in mollusc hemocytes.

Zymosan (a yeast surface protein) was used as the antigen for this assay and prepared at 13.5mg/ml in CBSS. The solution was boiled in an Eppendorff tube for 1 hour and then stored at 4ºC.

350µl hemolymph was added to the same type of Vaccutainer tube described in the encapsulation assay (but unaltered). Since this assay does not require direct visualization of cells it is not as important to obtain a single-cell suspension, such as for the flow-cytometry work. Therefore, Vaccutainers were used to allow interactions to take place in suspension and an equal volume of double-strength chemical solution in CBSS was added to the hemolymph and mixed.

For each assay two replicate tubes were prepared for each chemical/dose and 40µl zymosan solution was added to half the tubes in order to test for possible effects of the chemicals on both viability and phagocytosis. Table 5.1 provides possible scenarios and the subsequent predicted outcome.

Table 5.1. Proposed interaction between a test chemical and hemocyte function and the expected outcome this would indicate from the MTT assay.

Impact of chemical	Expected result from MTT assay
Chemical exposure reduces phagocytosis and not viability	Reduction in difference between zymosan and none zymosan when compared with control
Chemical exposure affects viability and not phagocytosis	Both with and without zymosan are lower than control but difference between them remains similar
If chemical exposure affects both viability and phagocytosis	Reduction in level of both zymosan and non-zymosan compared to control and less of a difference between zymosan and non-zymosan

Tubes were then incubated for 30 minutes in a water bath at 27^{O}C to allow interactions to begin, after which time 20µl of MTT solution was added to each tube (5mg/ml in CBSS). The interaction was allowed to take place for 4 hours after the addition of MTT. It is important to keep the tubes in the dark since the solution is photo-reactive.

After 4 hours, the suspension was centrifuged for 5 minutes at 1000g, at this stage it does not matter whether the cells are damaged/clumped.

The tubes were very carefully removed from the centrifuge so as not to disturb the pellet and the supernatant was poured off. During method development it became apparent that this step is the point at which most variability can be introduced to the assay since remaining hemolymph has the potential to cause a darker sample (and thus indicate a stronger response). Initially the supernatant was aspirated off but this method proved ineffective at removing all liquid without disrupting the pellet. The solution was to simply invert the tubes, but it was important to treat all tubes in the same manner and for this reason they were all placed in a large rack (Figure 5.8; A), secured, and then the rack itself was inverted, this way the supernatant from each tube was removed in the same way.

250µl DMSO was added to each pellet to dissolve the formazan crystals and the tubes were shaken for 20 mins to aid dissolution.

The tubes were spun once more for 5 minutes at 1000g and this time 200µl supernatant was added to wells of a 96-well plate (Figure 5.8; B) and absorbance readings were then taken at both 620 and 570nm using a plate reader.

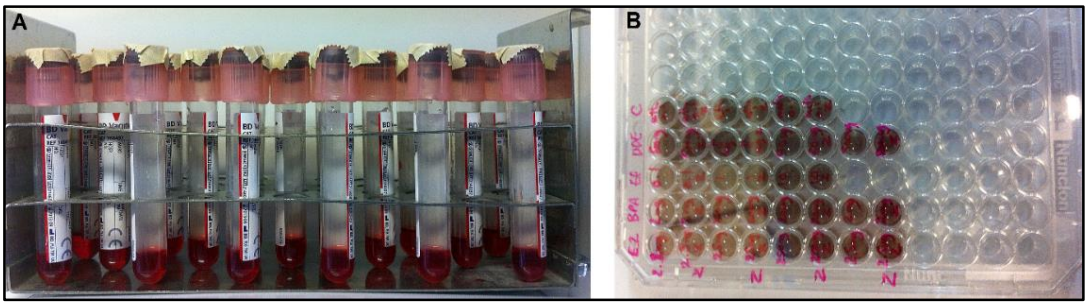


Figure 5.8. Example images of the setup for the MTT assay, adapted for studying phagocytosis in *B.glabrata*. *A) Vaccutainer tubes containing hemolymph, yeast and MTT and secured in a tube rack before incubation B) Samples after incubation and removal of supernatant with the DMSO-dissolved formazan crystals prepared in a 96-well plate for absorbance reading.*

5.2.6.2. *M.cornuarietis*

The following section describes the two *in vitro* phagocytosis assays which used hemocytes from *M.cornuarietis*.

5.2.6.2.1. Inhibitor dose-response; *in vitro* phagocytosis

Sodium azide (NaN_3), a known inhibitor of phagocytosis in mollusc hemocytes, was used to perform a dose-response experiment (Goedken and De Guise, 2004). Cells were prepared as a monolayer and exposed to NaN_3 in CBSS at 0, 0.5, 1, 1.5, 2, 2.5 and 3% in the presence of yeast particles (prepared as described in section 5.2.2.3). 5 minutes prior to use in the ImageStream an equal volume of 0.4% trypan blue was added to the sample and gently mixed in order to quench the fluorescence of extracellular yeast particles, thereby enabling more accurate determination of internalization from surface-adherence (Rodríguez *et al.*, 2003). Three individual

replicates were performed for each dose. 8000 cells were collected for each sample. Classifiers were set at: 100-500, 488: 20, 785: 1.

5.2.6.2.2. Effluent exposure assay; *in vitro* phagocytosis

I decided to perform a small study using the effluent extract as a means of assessing the suitability of *Marisa* in pollutant exposure assays and also as an opportunity to compare the response of *B.glabrata* hemocytes to those of another gastropod mollusc. The assay was performed in the same manner as for the *B.glabrata* hemocyte exposure to the effluent extract.

5.2.7. Statistical analysis

All dose-response data collected from the ImageStream was subsequently analysed using non-linear regression in a similar manner to that described in the previous chapter, with the exception of the *Marisa*-NaN_3 dose-response which followed a linear relationship. Number of beads per cell was treated as continuous in order to utilize the spot count data. Dichotomizing the response into positive and negative would only yield data on percentage phagocytosis and thus discard the extra information we have regarding the relative 'degree' of phagocytosis from the spot-count of each cell.

BioParticle data was analysed by t-test since the predictor was categorical with fewer than three categories (inhibitor presence/absence). Sensitivity and specificity of the masks ability to correctly identify and count internalized beads, when compared to human observation, was performed using Graphpad QuickCalcs. Intra and inter-pool variation were compared using percentage coefficient of variation. The MTT assay was analysed by multiple regression, with chemical dose and zymosan presence/absence as the continuous and categorical predictors respectively, and absorbance reading as the continuous response. Tests were performed in IBM SPSS version 22.

5.3. Results

In this chapter the results section is not only separated by individual assay but also by the two individual mollusc test species.

5.3.1. *B.glabrata; in vitro* phagocytosis assays

This section describes the results for *in vitro* phagocytosis assays performed with *B.glabrata* hemocytes.

5.3.1.1. Precision and accuracy

The variation in degree of phagocytosis (mean beads per cell) within replicates from the same cell pool (intra-assay precision) was calculated using equation 1 and resulted in a coefficient of variation of 6.6%.

$$\boldsymbol{EQUATION\ 1}: \quad \frac{\boldsymbol{Mean\ of\ standard\ deviation\ of\ replicate}}{\boldsymbol{Grand\ mean\ of\ replicates}}\ \boldsymbol{x\ 100}$$

Inter-assay precision, or variation in mean beads per cell, between *different* pools, was calculated using equation 2, and was 22.3%.

$$\boldsymbol{EQUATION\ 2}: \quad \frac{\boldsymbol{Standard\ deviation\ of\ the\ mean\ of\ replicates}}{\boldsymbol{Grand\ mean\ of\ replicates}}\ \boldsymbol{x\ 100}$$

Table 5.2 shows the performance of the custom counting mask compared with visual observation (i.e. the gold standard), in the form of a 2x2 contingency table. Fisher's exact test gave a two-tailed *P* value of 0.8281 indicating that there is no significant difference between the results given by the automated counting mask and those obtained by manually scoring individual cells.

Table 5.2. Performance of the custom mask based on observed (result from custom mask) vs expected (result from visual observation) values for phagocytosis classification.

	Phagocytosis (-)	**Phagocytosis (+)**	**Total cells**
Custom mask	372	130	**502**
Visual observation	376	126	**502**
Total cells	**748**	**256**	**1004**

P = 0.8281 according to Fisher's exact test

5.3.1.2. Bioparticle; *in vitro* phagocytosis assay

The Bioparticle assay, although relatively simplistic, was needed in order to test its suitability as a primary antigen for use in the later phagocytosis assays.

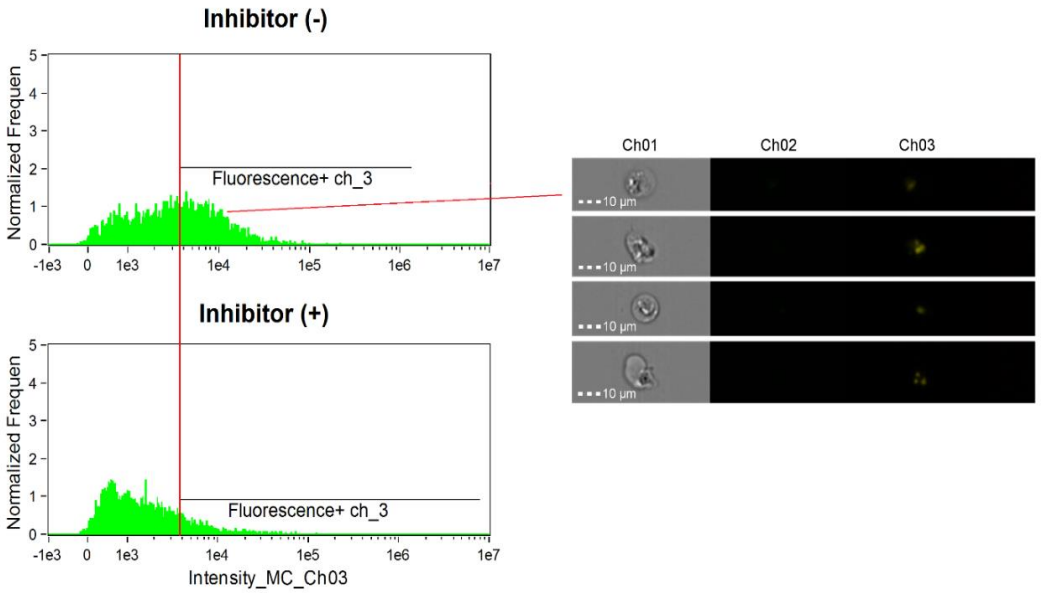


Figure 5.9. Frequency histograms of fluorescent intensity from Bioparticles exposed to hemocytes and incubated with or without phagocytosis inhibitor cytochalasin B. *Inhibitor (+) represents hemocytes and Bioparticles incubated with cytochalasin B and inhibitor (-) represents those without cytochalasin B. The population right of the red line represents cells which had successfully phagocytosed Bioparticles.*

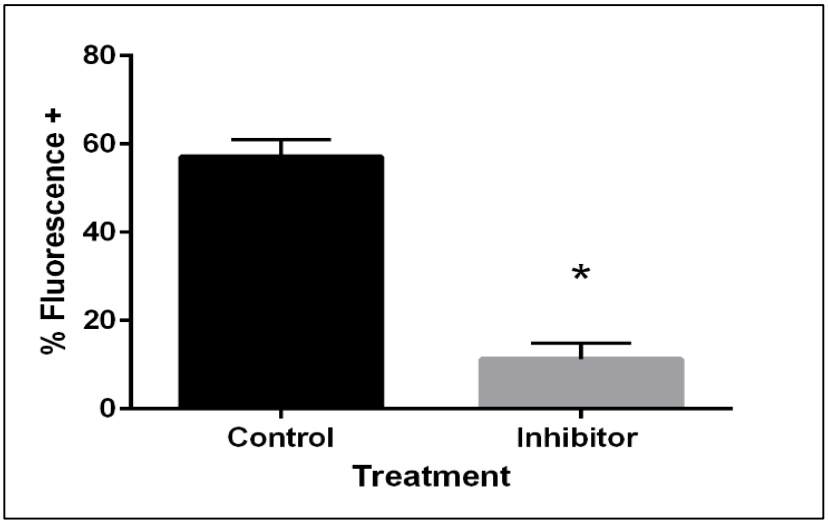


Figure 5.10. Level of phagocytosis as represented by the percentage of fluorescence positive cells from hemocytes incubated with or without the inhibitor cytochalasin B. *N =3, asterisk represents a significant (P = <0.05) difference when compared to control, according to one-way T-test.*

The reduction in the right tail of the histogram in Figure 5.9 (inhibitor +) represents a decrease in channel 3 fluorescence intensity when compared to the inhibitor negative sample (inhibitor -). Decrease in intensity compared to control is directly related to decrease in phagocytosis. The intensity data from Figure 5.9 can be translated into a significant difference between the inhibitor and control, with greater intensity representing greater levels of phagocytosis (Figure 5.10).

5.3.1.3. *in vitro* exposure of hemocytes to study chemicals

Given the positive response of the assay in detecting changes in phagocytosis in response to the inhibitor, I then tested the phagocytosis response of the hemocytes with a range of environmental chemicals and a domestic effluent extract (Figure 5.11).

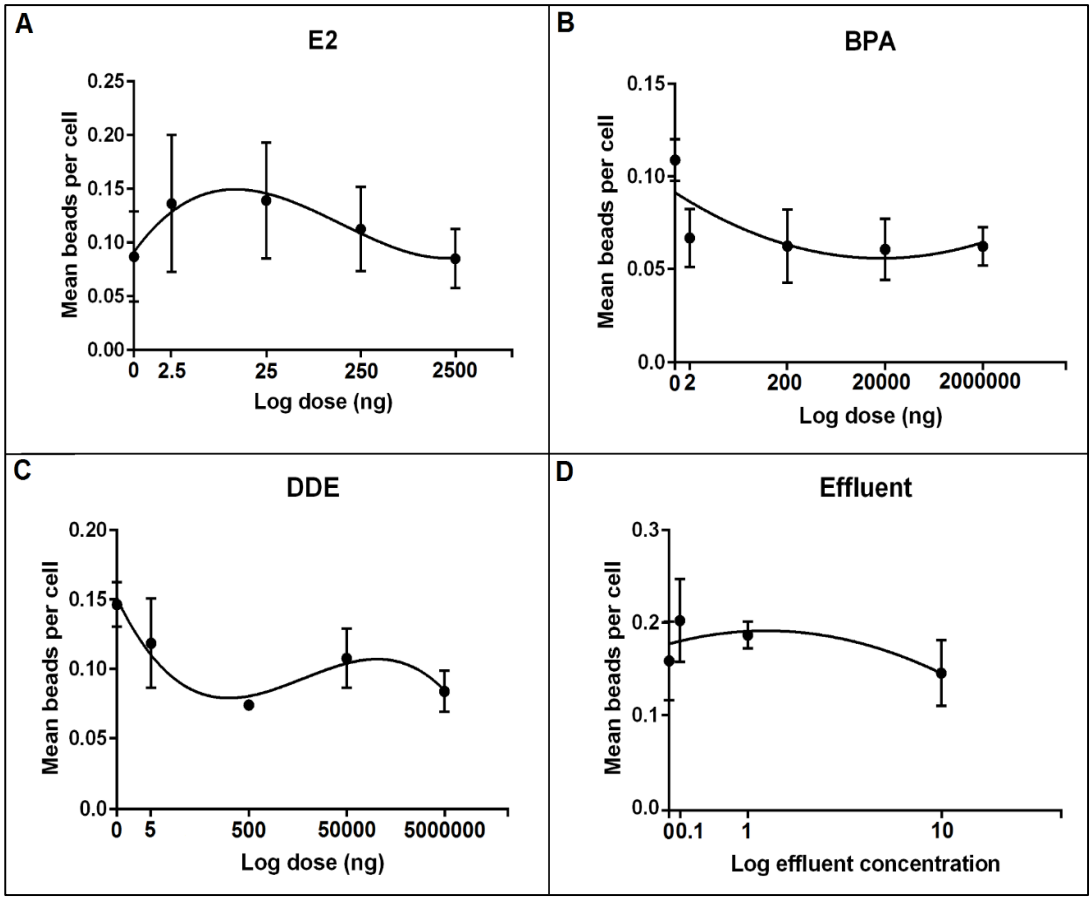


Figure 5.11. Regression relationships between log dose (X axis) of test chemicals against phagocytosis of latex beads by *B. glabrata* hemocytes exposed *in vitro* (Y). *Hemocyte response plotted as mean number of beads per cell ± SEM of 4 independent replicates. A) Hemocyte phagocytosis response to E2 exposure B) Hemocyte phagocytosis response to BPA exposure C) Hemocyte phagocytosis response to Effluent exposure D) Hemocyte phagocytosis response to DDE exposure.*

As with the encapsulation assays, all dose-response relationships were best fit by non-linear polynomials, with BPA and effluent following a second order (quadratic) curve and E2 and DDE following a third order (cubic). DDE dose showed the strongest ability to predict the value of mean beads per cell explaining >59% of the variation (Adjusted R^2 = 0.5943). The next strongest relationship was for E2, where 32% of the variation in mean beads per cell was explained (Adjusted R^2 = 0.32), closely followed by BPA dose which explained 31% of the variation (Adjusted R^2 = 0.31). Effluent concentration showed a very weak relationship with phagocytosis response, accounting for just 6.8% of the variation in the mean number of beads per cell (Adjusted R^2 = 0.06851).

The fundamental parameters of all models were appropriate (i.e. the fit was not ambiguous), there were sufficient data points and confidence intervals were not excessively wide, the normality test was passed and in each case the D'Agostino-Pearson omnibus K2 test was not significant, nor was the replicates runs test (p = >0.05).

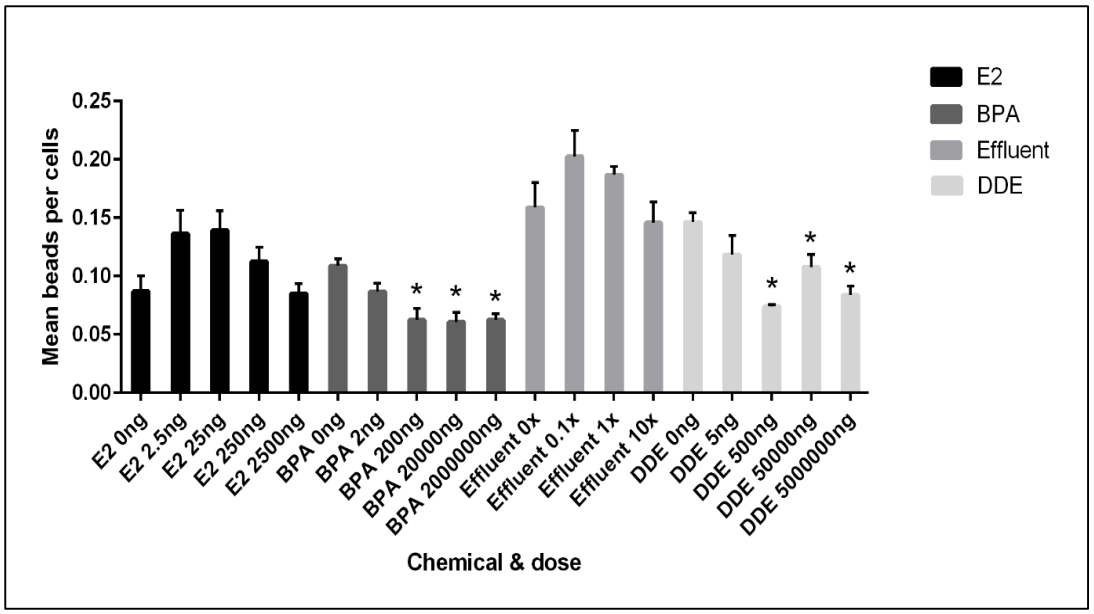


Figure 5.12. Multiple comparisons between mean number of beads per cell (X) at each test chemical and dose (Y). *Hemocyte phagocytosis response plotted as mean number of beads per cell ± SEM of 4 independent replicates for all doses of each chemical (Y). Response to doses of the same test chemical in different shades. Asterisks indicate doses which were significantly different to their respective control (p = < 0.05) according to Dunnett's test for multiple comparisons.*

Results from the multiple comparisons test (Figure 5.12) showed no significant difference from control for E2 and the effluent extract. Indeed, variability in the level of phagocytosis in hemocyctes exposed to each dose of E2 varied widely as shown in Figure 5.11.

For both BPA and DDE the responses at the highest three doses (200ng, 20µg, 2mg and 500ng, 50µg and 5mg respectively) were found to be significantly lower than their respective controls, indicating a degree of inhibition in phagocytosis response at these doses.

5.3.2. *M.cornuarietis*

Trypan blue proved to be extremely effective at quenching fluorescence of extra-cellular yeast, enabling clear visualisation (fluorescence) of cells containing internalized yeast on Channel 2 (Figure 5.13; A) and non-internalized yeast showing extremely low Channel 2 fluorescence (Figure 5.13; B). This enabled me to effectively gate phagocytosis positive cells based on Channel 2 intensity (Figure 5.13).

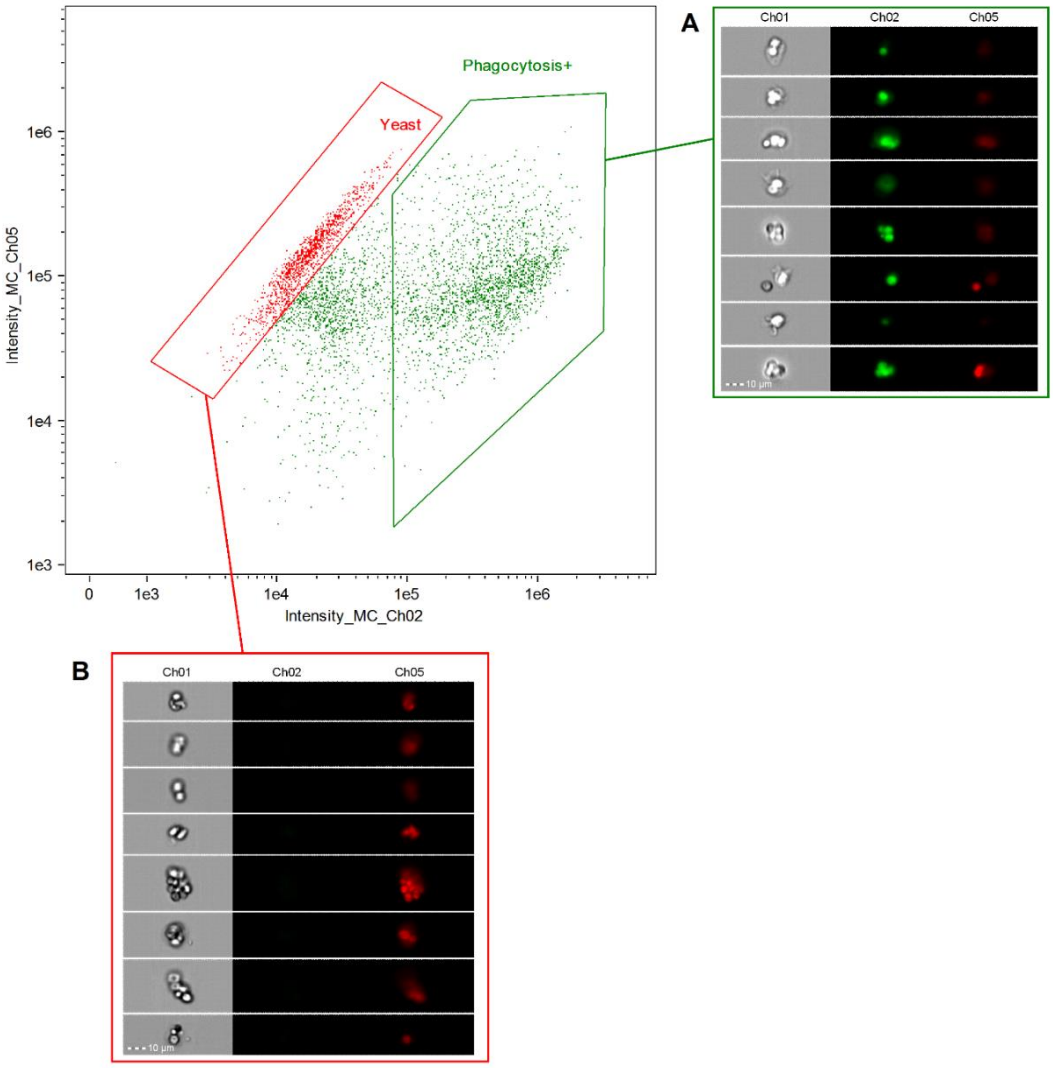


Figure 5.13. Scatter graph showing phagocytosis of yeast by *M.cornuarietis* hemocytes and the ability to separate out yeast clumps based on fluorescent profile**.** *Points plotted represent fluorescence intensity for channel 2 vs channel 5. Green gated region represents hemocytes positive for phagocytosis of yeast as shown by image A. Red gated region represents yeast clumps as shown by image B.*

An unexpected, but very beneficial, outcome was the finding that the yeast cells displayed a strong red fluorescence independent of quenching (Channel 5; Figure 5.13 A and B). This meant that the unavoidable yeasts clumps which passed through the sample could easily be gated out based on their high Channel 5/low Channel 2 intensity. The yeast clumps that came through were approximately the same size and shape as the hemocytes and therefore could not be filtered or gated out based on size alone, as would typically be the case. Their tendency toward strong C5 fluorescence meant that separation and categorization of all objects in the sample was achieved based on the criteria shown in Table 5.3.

Table 5.3. Criteria used for differentiation of objects based on their different Channel 2 and Channel 5 intensities.

	Channel 2 intensity	Channel 5 intensity
Phagocytosis (+)	↑	↑
Phagocytosis (-)	↓	↑
Extra-cellular yeast clump	↓	↑
Dispersed external yeast	**No signal**	**No signal**

The green (Channel 2) signal is only observed when yeast are inside the cell, since it is quenched by trypan blue if it is not protected by a cell wall. Internalised yeast cells are therefore distinguished from external yeast clumps by a simultaneous strong Channel 2 and Channel 5 signal, whereas external yeast clumps appear on Channel 5 alone. Non-phagocytosisng cells display a channel 2 intensity lower than phagocytosis positive cells but higher than yeast clumps, possibly due to a degree of autofluorescence on channel 2. Dispersed yeast cells are effectively quenched and are not visible on either channel (Table 5.3).

What became immediately apparent when performing assays using *Marisa* hecomcytes was that they were considerably easier to work with than *B.glabrata*.

Marisa cells produced lower intra and inter pool coefficients of variation (4.2% and 9.5%, respectively) than *B.glabrata*.

Marisa cells responded more consistently in most aspects (e.g. spreading rates, phagocytosis percentages and number of objects internalized), however *Marisa* showed a very poor response to the latex beads, in much the same way that *B.gabrata* responded poorly towards the yeast. Comparatively, the *Marisa* cells ingested many more of their 'preferred antigen' than *B.glabrata* i.e. the response of *Marisa* hemocytes to yeast cells appeared to be stronger than the response of *B.glabrata* hemocytes to latex beads.

The spot count mask designed for use with *B.glabrata* hemocytes and fluorescent beads was modified slightly for Marisa, to accommodate the difference in antigen size and fluorescence intensity, and was similarly effective in its ability to correctly count intra-cellular objects (Figure 5.14). As with the *B.glabrata* mask, no zero counts were observed in the selected data set as would be expected for a precise mask applied to an entirley phagocytosis positive population.

The ability of the mask to exclude non-internalized yeast was relatively unimportant (unlike latex beads) since the yeast signal is amenable to quenching and so internalization status was based on fluorescence intensity.

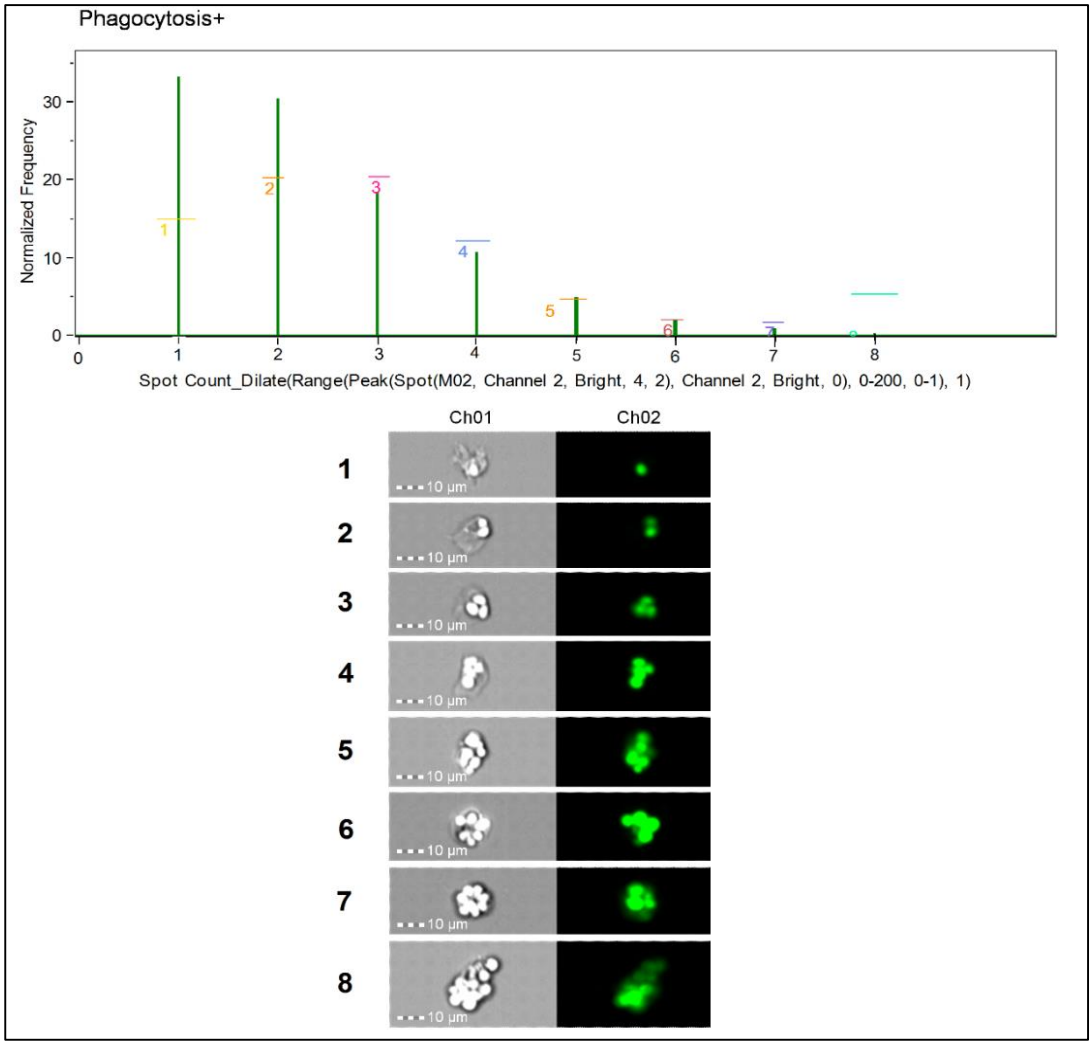


Figure 5.14. Frequency histogram of the number of yeast cells inside phagocytosis positive *M.cornuarietis* hemocytes according to the spot count mask. *The image gives an example of hemocytes containing between 1 and 8 yeast cells, as seen in both the brightfield and in channel 2.*

5.3.2.1. Inhibitor dose-response; *in vitro* phagocytosis

While there is no information in the literature regarding the use of NaN_3 as a phagocytosis inhibitor of *Marisa* hemocytes, it was evident from the strength of the dose-response relationship that this chemical can be applied to *Marisa* hemocytes for the same purpose (Figure 5.15), thereby serving as a viable positive control.

Linear regression established that NaN_3 dose was highly statistically significantly in its ability to predict the mean number of yeast internalized per hemocyte, F (1, 12) = 49.2, p = < 0.0001 and accounted for 80% of the explained variability (r^2 = 0.8039). The linear regression equation was: Mean number of yeast per hemocyte = -0.2735*(% NaN3) + 0.9329.

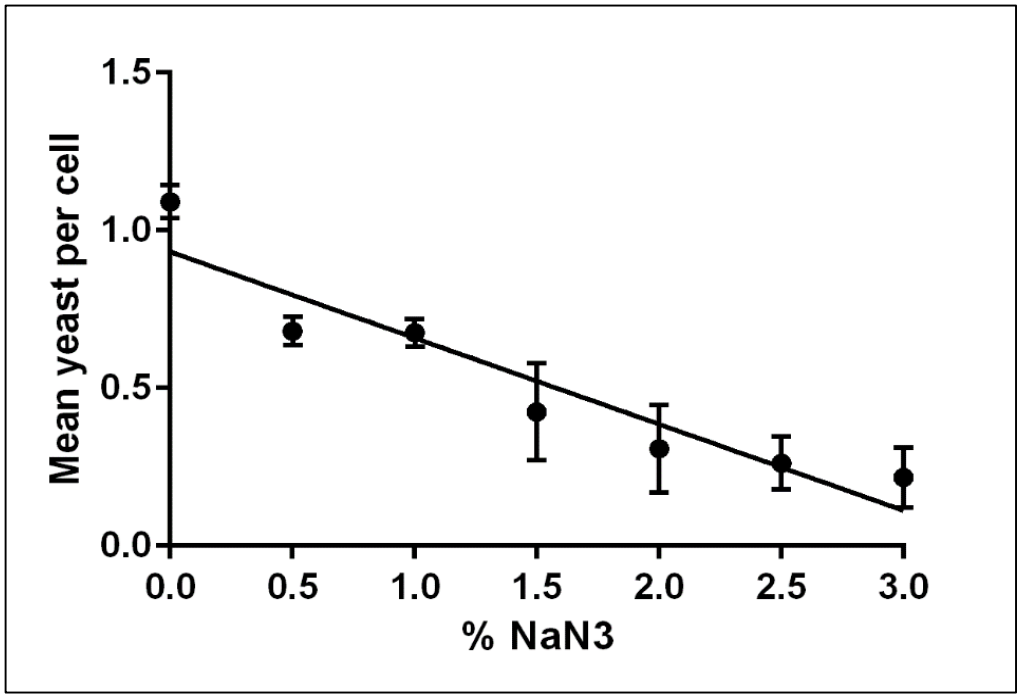


Figure 5.15. Regression relationship between phagocytosis response as measured by mean number of yeast per *Marisa* hemocyte (Y axis) and the percentage of NaN_3 to which it was exposed (X axis). *Data points represent mean number of yeast per cell ± SEM of 4 independent replicates.*

5.3.2.2. Effluent exposure assay; *in vitro* phagocytosis

Based on the promising results obtain from the preliminary assays and the apparent reliability of the phagocytosis response in *Marisa*, I decided to test the Marisa hemocytes against the effluent extract to determine if the response was more profound, and the cells more sensitive to effluent, than with *B.glabrata* (Figure 5.16).

As with *B. glabrata* (Figure 5.11; D), the response of *Marisa* hemocytes to effluent extract was very low and effluent concentration was only able to explain 2.3% of the observed variation in the mean number of yeast per cell (Adjusted $r^2 = 0.02302$). The shape of the relationship was relatively similar to that of *B.glabrata* with the best fitting model also being a Second order polynomial but the magnitude of response was considerably greater on average.

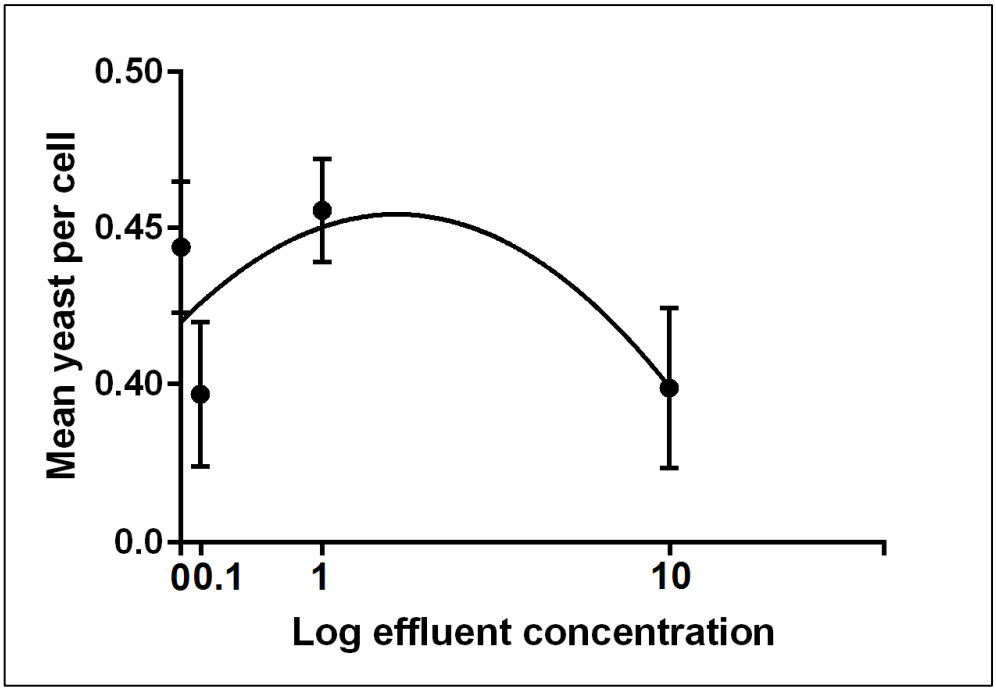


Figure 5.16. Regression relationships between log dose (X axis) of effluent extract against phagocytosis of yeast by *Marisa* hemocytes exposed *in vitro* (Y). *Hemocyte response plotted as mean number of yeast per hemocyte ± SEM of 4 independent replicates.*

5.3.2.3. MTT; *in vitro* phagocytosis assay

In each case, multiple regression analysis on the MTT assay data revealed that there was a statistically significant relationship between the X variables (dose or zymosan status) and the Y (absorbance) variable (P = <0.01) and that the model could explain a maximum of 28.06% of the variation in absorbance reading (R^2 = 0.2806). However, the outputs revealed that the vast majority of the contribution to increase in R^2 was due to the addition of zymosan and not associated with the dose of any of the test chemicals. Since the addition of zymosan is expected to cause an increase in phagocytosis, the significant contribution of Zymosan to the model is expected. The results therefore show that zymosan is effective at stimulating the phagocytosis response, but that none of the chemicals/doses had any significant influence on this response in this particular assay. This is also evidence that the test chemicals did not induce significant cell death in any of the doses, something which was previously established in preliminary tests.

5.4. Discussion

The study of phagocytosis in molluscan hemocytes is an innately challenging process. Quantitative phagocytosis assays are well known to display considerable *intra*- and *inter*-assay variation regardless of the species for which they are used (Rodríguez *et al*., 2001). This is potentially exacerbated by the fact that biological variation in primary cell cultures is typically considerably higher than in cell lines (Molloy *et al*., 2003). Another considerable challenge is the limitation in cell quantities that can be obtained from *in vivo* sources, thus limiting throughput. For these reasons, it is not surprising that the vast majority of phagocytosis studies in the scientific literature are performed using established cell lines with predictable properties and which can be easily cultured to yield a desired number of cells. These difficulties are apparent even without the added complications provided by molluscan hemocytes. Nevertheless, it is possible to develop assays to study the process of phagocytosis in such cells if their particular nature is taken into account and procedures adapted accordingly.

One way in which cell yields can me optimised, and high levels of variability reduced, is through pooling of material from individual animals. The precision (i.e. the closeness of agreement between replicated and independent test results) of phagocytosis response within and between hemocyte pools was measured in an attempt to understand the variation present within my test samples (Pryseley *et al*., 2010). Such measurements are typically made using the coefficient of variation (CV) within and between the samples. Standard deviation of such assays generally increase or decrease proportionally as the mean increases or decreases and division by the mean removes this as a factor in the variability measurement. The main appeal of the CV, therefore, is that it is a normalization of the SD that enables comparisons of variability estimates regardless of the magnitude of response.

There is no set definition of an acceptable criteria for the CV, and interpretation of the results depends on the hypothesis, the type of assay, the techniques used and most importantly; the nature of the sample. Despite this, there are some broad definitions of ‘acceptable’ (or more accurately ‘desirable’) values. *Intra* assay CVs of ≤10% and inter assay CVs of ≤20% are desirable, but in some cases an *inter*-assay CV of up to 50% may be accepted (Murray *et al*., 1993).

Given the nature of *B. gabrata* hemocytes, the measured *intra* and *inter*-pool CVs for phagocytosis of 6.6% and 22.3%, respectively, were considered satisfactory for my uses. *Intra*-assay values would almost always be expected to be lower than *inter*-assay values since the cells being studied were from the same population.

There is one other study in which *intra* and *inter*-pool CVs for molluscan (oyster) hemocytes had been calculated based on their ability to internalize latex beads. Hegaret *et al*., (2003) found within- and between pool variations of 21% and 18% respectively; an interesting result since the *inter*-pool variation was lower. They concluded that pooled samples could be successfully used to represent the mean and variance of mollusc populations contributing to the pool (Hegaret *et al.*, 2003),

Another approach used to improve confidence in my assay and its results was to assess the sensitivity and specificity of the measurement; something that is often neglected in phagocytosis studies.

One of the key advantages of imaging flow cytometry is the ability to fuse the sensitivity of microscope visualisation with the high-throughput nature of standard flow cytometry *via* the collection of detailed images for every data point. There is often a tendency to apply high-tech systems for their own sake, but to truly take advantage of ImageStream flow cytometry it is important to realise that both throughput and the accuracy of counting/scoring cells is superior to traditional methods. Indeed, if the counting method is not accurate then there is little merit in obtaining very large amounts of poor quality data, in which case counting a smaller number of cells by standard microscopy would be a better option.

To date there are no published examples of imaging flow cytometry using mollusc hemocytes in any capacity. Phagocytosis in *B. glabrata* was only recently reported using standard flow cytometry by Bakry and colleagues (Bakry *et al*., 2012b). The majority of phagocytosis studies in molluscan hemocytes have quantified effects based on visual examination of cells under light microscopy.

Visual observation of antigens (for example yeast, beads etc.) inside hemocytes is clearly a strong indicator of phagocytosis, but quantification is extremely time consuming and labour intensive. On the other hand, standard flow cytometry is capable of examining hundreds, even thousands, of cells per second, although

identification of phagocytosis is less straight forward since it relies on instrumental determination based on set properties other than judgment by the human eye.

While gating of cells based on their morphological and/or fluorescent properties is generally effective, it is never possible to be certain that the gating process is entirely accurate without visual examination of the cells. As a result, many studies using standard flow cytometry rely on defining phagocytosis as 'objects with *three or more beads*' since sensitivity in detecting between cells with no beads and cells with a single bead is very difficult based on fluorescence alone, especially at the boundaries where the internalized object may be vague, for any number of reasons, and the phagocytosis negative cell may have a high degree of auto-fluoresence. It is in these situations that imaging flow cytometry offers significant advantages over traditional methods, especially since my findings, and those of others, show that the majority of phagocytosis positive cells do contain only a single bead and would therefore normally be excluded from analysis resulting in potentially inaccurate findings (Luengen, 2004; Parra *et al*., 2012).

It is extremely important to know that automated counting methods are comparable to manual observations in order for researchers to take full advantage of these technologies whilst being reassured that they would not have been better off using standard microscopy. I observed a high level of agreement between results of manual counting (i.e. what could be considered 'correct' according to the gold-standard) and automated counting using the mask I developed (section 5.2.5). This means that of the thousands of cells measured, I would not have been significantly more accurate by counting each cell manually ($P = 0.8281$; Table 5.2).

In the context of automated counting methods, there are two types of antigen which can be considered: those that are most amenable to precise automated counting and those that are easier to classify as phagocytosis positive or negative. For example, Bioparticles (*E.coli* with conjugated PHrodo stain) were investigated due to their unique properties. These antigens can be used directly to determine truly phagocytosis positive cells. Normally this distinction is one of the more difficult aspects of the assay and may require a number of experimental steps during the preparation of the cells (e.g. filtering out non-internalized objects) or afterward (e.g. gating, masking out). In principle, if a Bioparticle appears fluorescent then the cell is

considered '*phagocytosis positive*' as the dye fluoresces only upon acidification of ingested particles within maturing phagosomes. In this way, fluorescence indicates that *E. coli* has been internalized rather than remaining attached to the surface of the cells (Parra *et al*., 2012). My research showed that the Bioparticles were amenable to *B. glabrata* hemocytes and the addition of cytochalasin B (a known inhibitor of *E.coli* phagocytosis) caused a significant decrease in fluorescence intensity (Figure 5.10).

Despite this, there were some disadvantages in using Bioparticles, which meant that latex beads were generally preferred for my work. The first consideration was the high price of Bioparticles, which retail at around £120 per 2mg; an amount that would cover a relatively small number of assays. In contrast, latex beads are much cheaper, owing to their more simplistic manufacture and the fact that they are developed by, and can be purchased from, numerous different companies. From a purely scientific perspective, the main drawback of Bioparticles was the fact that less information was obtained due to their significantly lower intensity compared to latex beads, as well as their less uniform shapes (Figure 5.9); both these factors made the signal-to-noise ratio lower and development of accurate counting masks very difficult. Masks are not essential to obtain data regarding percentage phagocytosis as this can be based on fluorescence intensity of individual cells alone. However, I wished to assess the 'degree' of phagocytosis per cell rather than a simplistic 'positive or negative' count.

The phagocytosis dose-responses showed some moderate similarities to those of the encapsulation assays, possibly due to the similarities in the mechanisms that govern both responses. Phagocytosis responses following exposure to various doses of E2, BPA and the effluent extract were all best modelled by third-order polynomials, as was the case for encapsulation response. DDE was the only chemical to show a markedly different shaped dose-response between phagocytosis and encapsulation (third-order polynomial vs first-order respectively). The strength of the relationships were also relatively similar, as were the results for Dunnett's test when comparing against the control. As with the encapsulation assays, certain doses of BPA and DDE produced responses that were significantly different from the control group. For the encapsulation assays only the highest doses of BPA and DDE significantly inhibited the response compared to the control (Figure 4.5). However, all but the lowest dose

of these chemicals (200ng-2mg; BPA and 500ng – 5mg; DDE) significantly inhibited the phagocytosis response compared to the controls, suggesting that the phagocytosis assay is perhaps more sensitive than the encapsulation assay using these chemicals.

An exception to the general similarities between the encapsulation and phagocytosis responses was observed with the effluent treatment, which showed a moderate to high coefficient of determination when predicting encapsulation but a very weak one when predicting phagocytosis. One possible explanation for the observed differences between these responses may be the different antigens used i.e. latex beads rather than sporocysts which are biological and have surface antigens. It has been shown that there may be differences in the responses of cells to different phagocytosis antigens under the influence of certain stressors. For example, Parra and colleagues found that the phagocytosis response of human immune cells to *E. coli* and latex beads was altered (reduced and unchanged, respectively) following infection by rhinovirus (Parra *et al*., 2012). Differences in reported responses of immune cells to different antigens was one of the reasons that I tested different antigens in the response to the effluent extract.

My data suggests that *Biomphalaria* hemocytes are not amenable to use with yeast in the flow cytometry assays. The hemocytes failed to behave in a manner that allowed a single-cell suspension to be obtained and instead formed clumps around the yeast – this is a phenomenon which has previously been reported when using yeast (Connors and Yoshino, 1990). Phagocytosis is occurring inside the cell clumps but the response is closer to that of encapsulation, perhaps due to the large size of the yeast cells compared to beads and bacteria (Connors and Yoshino, 1990). Since *Biomphalaria* hemocytes reacted poorly to the yeast and *Marisa* hemocytes reacted poorly to the beads, it was necessary to test the effluent extract against *Marisa*/yeast to see how the response might differ under different circumstances.

Marisa hemocytes displayed a similarly weak response to the effluent extract (Adjusted R^2 = 0.02302). Given that (i) the response between phagocytosis rate and effluent dose was similar in hemocytes from a different snail species using a different antigen, and (ii) that phagocytosis and encapsulation are very closely related mechanisms, it would appear that *B. glabrata* hemocytes only respond

differently to sporocysts in the presence of the effluent extract, for this was the main difference between the two assays that could not be accounted for. In other words; the effluent extract may cause a degree of modulation in the immune response of snail hemocytes to sporocysts but not necessarily to other antigens.

Aside from the response of snail hemocytes to the effluent extract (which is entirely novel) the results of dose-response assays using pure test chemicals were also relatively difficult to interpret as there are so few studies investigating mollusc immune responses to these chemicals and more specifically involving *B. glabrata*. Nevertheless, there is generally a little more information available regarding phagocytosis compared to other types of immune response.

Both E2 and BPA have generally been found to have a stimulatory effect on mollusc hemocyte phagocytosis at low (environmentally relevant) concentrations *in vitro* and inhibition at higher concentrations (Canesi *et al*., 2006; Canesi *et al*., 2007a). This non-monotonic dose response shape was somewhat evident in E2, with the first three doses appearing to have a higher mean response compared to the control, although the differences were not significant (Figure 5.12). It should be noted that in other reports the low dose stimulation often took longer (~6 hours) to occur than higher dose effects (Canesi *et al*., 2006), and this time frame was longer than that used in my assays.

Comparisons with the literature are further complicated by the fact that several studies are based on hemocytes collected from animals that have been injected with test chemical, after which the cells are quickly removed for analysis. In these cases, information is given regarding the quantity of the chemical injected into the animals, but the precise level of systemic exposure to the cells is not clear (Canesi *et al*., 2006; Canesi *et al*., 2007a).

Canesi et al., (2006) report one possible mechanistic reason for the immune suppressive effects observed with estrogenic chemicals, something which was relatively pronounced with higher doses of BPA in my study (Figure 5.12). E2 and BPA are thought to impair the mechanism involved in controlled membrane fusion events during the phagocytic process (Canesi et al., 2006). Further evidence that lysosomal membrane destabilization assays follow the same pattern supports this contention (Canesi *et al*., 2006). The same authors suggest that EDC-induced effects

on the mollusc ERK/p38 MAPK signalling pathways, which are of major importance in the regulation of phagocytosis, may also represent a major contributing factor (Canesi *et al.*, 2006).

After establishing the most suitable antigens for use with Marisa hemocytes, a dose-response curve was performed against sodium azide (NaN_3); an established inhibitor of phagocytosis in hemocytes and immune cells in many species. To my knowledge, the response of *Marisa* hemocytes to NaN_3 (to establish a phagocytosis response relative to a negative control) has not been reported in the literature, so this assay represented an important first step. The *Marisa* hemocytes displayed a classic strongly negative dose-response ($r^2 = 0.8039$) to NaN3 (Figure 5.15) which is a strong indicator of their suitability for use in assays to assess effects induced by other chemicals.

Although I hoped to use exactly the same techniques with *Marisa* hemocytes as those developed for *B.glabrata* (enabling direct comparison of immune responses in two species of mollusc), the specific nature of the Marisa cells required some minor alterations, most notably the use of yeast instead of beads.

The yeast had both advantages and disadvantages when compared to the latex beads. The primary disadvantage of using yeast was its tendency to form extremely strong clumps. While these clumps could be broken apart to a considerable degree, it is not uncommon for small clumps of around 5-10 cells to remain. These clumps are approximately the same size as the hemocytes, making gating a much more difficult task compared to the far smaller latex beads (which themselves do not have a propensity toward clumping). The main advantage of using yeast cells (apart from their low cost) was the ability to quench the fluorescence of non-internalized cells using trypan blue. This is possible because the dead yeast have compromised cell walls that allow trypan blue to enter and 'turn off' the green autofluorescence of the entire cell, whereas those which have been internalized are protected from the dye by the intact cell wall of the hemocyte they reside within. The ability to quench the unwanted signal meant that a complex masking strategy (such as the one developed for the beads) was unnecessary. Additionally, not only did quenching help to differentiate between phagocytosis positive and negative cell, but it also meant that the large cell clumps could be gated out based on their differential fluorescence

profile when compared to all other populations of interest (Table 5.3). This represents an added advantage to the use of flow cytometry over other reported methods for investigating phagocytosis. At the time these assays were performed, the application of this masking approach in imaging flow cytometry was novel. However, since then at least one paper has been published which makes use of a similar approach (Avelar-Freitas *et al.*, 2014).

While the *Marisa* hemocytes appear to respond in a similar manner to *B. glabrata* (e.g. to the effluent extract), the average response was typically at least twice as high (Figure 5.11 D and Figure 5.16). This is supported somewhat by the findings of Yousif and Lämmler (1975) who showed that *Marisa* hemocytes display a considerably more vigorous defence response than *B. glabrata* to the parasitic nematode *A. cantonensis;* even though species both snails are effectively non-compatible with.

In general, *Marisa* appears to be an excellent candidate as a model species in the investigation of *in vitro* phagocytosis. The species displays numerous physical traits which make it superior to certain other gastropod molluscs. In particular, their large size, ease of husbandry, high hemocyte yields, light coloured hemolymph (which could, for example, aid in its use in colorimetric assays) and the ability to study the effects of EDCs on separate sexes, are obvious advantages.

While it was never intended to focus on *Marisa* in this project, my data shows that it offers considerable potential as a model species for studying the effects of environmental contaminants on gastropod molluscs. The species itself is not of particular economic or medical importance, yet it is likely that work performed on this species would be applicable to medically important gastropods which themselves are much more difficult to study.

In general, the results from the phagocytosis assays yielded responses which were reasonably consistent with the few reported examples in the literature and also with the encapsulation assays which mechanistically are likely to be very similar. While the effluent extract did not appear to have any clear relationship with phagocytosis at the tested doses, the difference in outcome with the encapsulation assay and the possible reasons for this warrant further research.

Chapter 6: *Cell Motility; development of a low-cost assay with wider applications*

6.1. Introduction

Cell motility has become an integrated measure used in a variety of modern assays spanning many research disciplines. Motile cells, individually or as groups, are vital to biological processes including fertilisation, growth and differentiation, immunity and the progression of diseases, such as cancer (Meijering *et al*., 2009; Huth *et al*., 2010; Hulkower *et al*., 2011). Consequently, a number of systems for studying motility are available to researchers and practitioners. For example, Trans-well assays (such as the Boyden chamber) enable measurements of cell motility in response to a chemical stimulus (chemotaxis). Chemotactic responses are quantified by the extent to which cells will migrate across a porous membrane towards a chosen test chemical (Dai *et al*., 2005) However, despite being a relatively inexpensive means of measuring motility Trans-well assays do not permit direct observation of cells as they move (Dai *et al*., 2005; Pujic *et al*., 2005).

Direct visualisation is considered to be the 'gold-standard' in motility studies, enabling precise and continuous measurements of the speed, trajectory and morphology of individual cells under a microscope (Muinonen-Martin *et al*., 2013). Cell motility is recorded by equipping the microscope with a digital camera and acquiring pictures at specific intervals over a chosen period of time (time-lapse) (Huth *et al*., 2010). However, individual experiments often take several hours, and possibly days, to complete making these a daunting and laborious task when only a single microscope is available.

Over the last couple of decades, there have been considerable technological advancements in microscopy hardware and software to enable a large degree of automation over time-lapse studies (Zanella *et al*., 2010; Buggenthin *et al*., 2013). These advances include motorised stages and auto-focusing software which together allow the acquisition of images across numerous samples without the need for user intervention (Zanella *et al*., 2010). A basic commercial setup for performing motility studies usually requires an inverted microscope, digital camera, software and an incubator/heated stage and would be expected to cost several thousand pounds (GBP) whereas many automated systems can reach several hundred thousand pounds (Gough and Johnston, 2007; Zimic *et al*., 2010). Due to high software and hardware costs such systems are accessible only to those with large budgets, and are

often outside the reach of non-specialist researchers, or those in developing countries with fewer resources (Buggenthin *et al*., 2013). Indeed, affordable solutions for the direct visualisation of cells is rapidly becoming a research area in its own right (Bishara *et al*., 2011; Smith *et al.*, 2011; Kim *et al*., 2012; Linder *et al*., 2013).

The recent ability to produce low-cost imaging devices is a consequence of improvements in image sensors such as charge coupled devices (CCDs) and complementary metal oxide semiconductors (CMOS) enabling good quality imaging together with substantial decreases in size and cost (Kim *et al*., 2012). Such devices are now produced for a range of applications from disease diagnosis to measurement of sperm motility (Su *et al*., 2010; Bishara *et al*., 2011; Linder *et al*., 2013). However, although these can vastly increase the affordability of motility assays and deliver high image quality, none can match the high-throughput nature of the more expensive devices. Therefore, the next stage in low-cost imaging will be to develop solutions to increase their throughput ability.

Along with advances in hardware, recent years have also seen a significant increase in the amount of high quality open-source software (which in many cases is comparable in capability to commercial packages) written for the analysis of microscopy data (Patlak, 2010). Here I show that by combining a number of low-cost imaging devices with open-source software we are entering a stage where high-throughput digital microscopy imaging can be an affordable option for all researchers. Building on existing work in low-cost imaging, and inspired by my desire for an affordable method to study cell motility in the context of xenobiotic exposure, I hope that this system will help make motility measurements accessible to all researchers, and inspire others to continue to enhance the productivity of similar devices.

6.2. Materials and methods

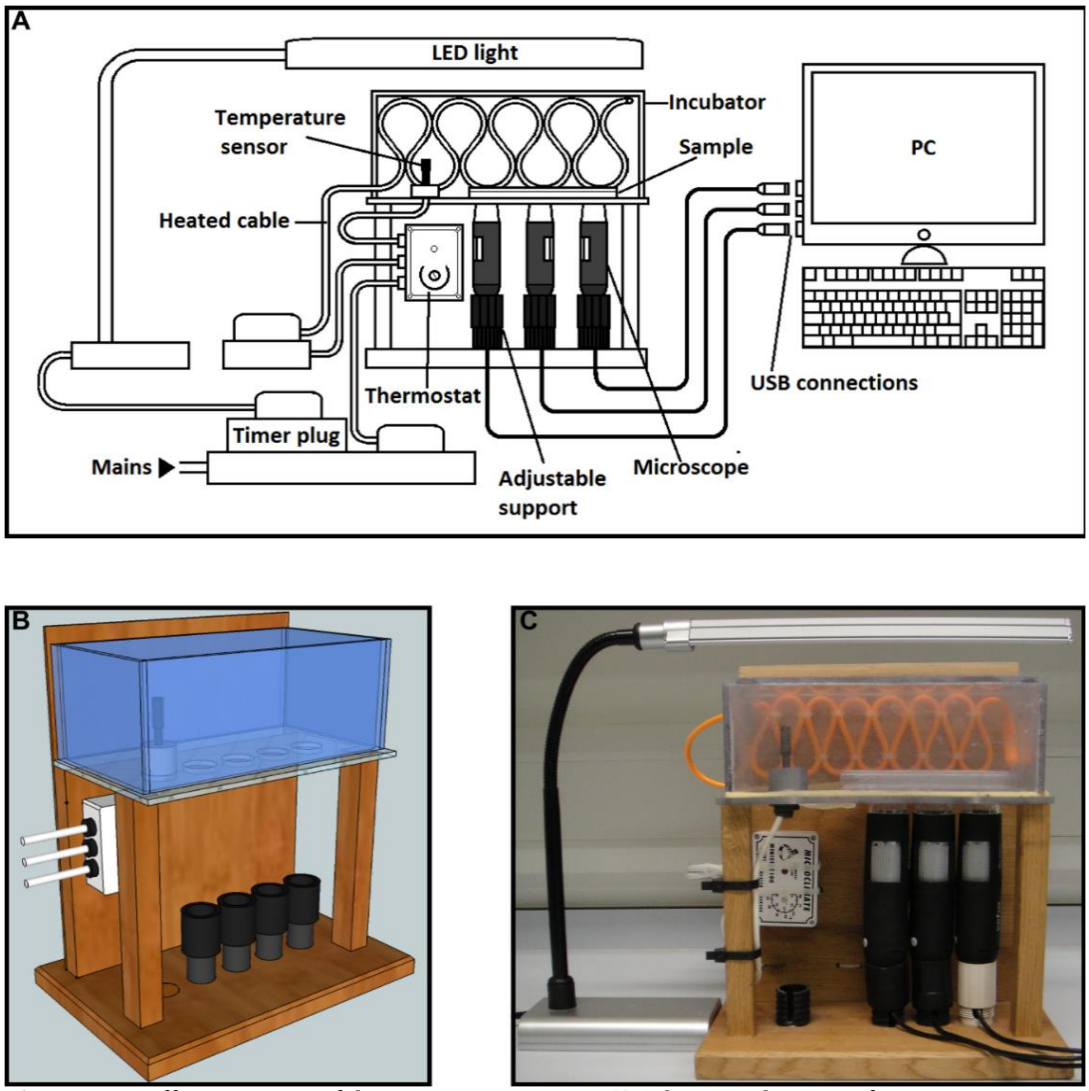


Figure 6.1. Different images of the microscope system. *A) Schematic diagram of microscope system B) CAD model C) Photograph of finished system.*

6.2.1. Construction and Calibration of the microscope system

The design process (Figure 6.1A) began by creating a 3D model of the system in Trimble Sketchup 8 (Figure 6.1B). The frame was constructed using oak, the stage from acrylic and the microscope support bases were created from adjustable kitchen unit legs, which fit them well and allow the height to be adjusted so that the top of the microscope is level with the stage and secure which prevents image drift and allows for optimum magnification (Figure 6.1C). Detailed information regarding the construction process can be found in the supporting information section of the published version of this chapter:

(http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0103547 ; Protocol S1, Protocol S2, Figure S1, Figure S2 and Table S1).

6.2.1.2. Microscopes

All three microscopes used were identical models (VMS-004D, Veho, Hampshire, UK) in order to prevent any discrepancies. These microscopes use a CMOS image sensor with 1.3 mega-pixel resolution (2MP with interpolation, although interpolation was not used in my studies). Magnification has two set levels (~ 20x minimum and ~ 400x maximum) achieved using a focusing wheel. To enhance stability, magnification and to allow for observation of live samples in liquid (cells) the microscopes were inverted.

6.2.1.3. Lighting

Various lighting sources were tested, and ultimately an LED strip desk lamp was selected. The microscopes' inbuilt LEDs were turned off. LEDs were used due to their low heat emission and intensity which helps reduce stress to the cells. To further prevent the risk of phototoxicity an accurate timer plug was used to turn the light off between image capturing. It was important to have a light source which could be adjusted since different samples have different requirements (due to depth or transparency).

6.2.1.4. Heating

In order to keep samples at a constant physiological temperature, without needing to place the whole system inside an incubator, an incubation chamber was developed to fit over the top of the stage. The chamber was made from transparent acrylic to allow visualisation inside. The edges of the chamber were fitted with foam to improve insulation and the chamber was secured above the stage using metal clips. The heating element used was a 37.5w soil warming cable; this was chosen since it is economical, waterproof and highly flexible. To maintain a constant temperature, the heating cable was connected to a commercial mini thermostat of the kind typically used to heat vivariums (MicroClimate Ministat 100). The stability of the incubator temperature was measured at 27^{o}C by placing a 'Tinytag' data logger (Gemini Data

Loggers, Chichester, West Sussex, UK) inside which was programmed to take temperature measurements every minute for 20 hours.

6.2.1.5. Resolution

The ultimate definition of resolution is described as the minimum distance two objects can be separated by and still are distinguished as separate features; this is sometimes referred to as spatial resolution. Spatial resolution is therefore the most important quantitative measurement. It is important to note that in digital systems spatial resolution is also related to, but not dependent on, pixel resolution which refers to the number of pixels utilised to create the image (width x height). In the case of digital imaging systems, it is therefore also necessary to consider pixel resolution, as this can determine the amount of information from the real object that is retained within the digital image. If the number of pixels used to create the image is too low information is lost.

Pixel resolution in Autokams was set at 640x480. Although the microscopes are capable of higher resolution, time-lapse studies typically generate a large number of files. Therefore, given the fact that three cameras were running together, a lower pixel resolution was chosen to reduce memory demand and enhance system stability. Spatial resolution was tested using 10µl of a 0.1% solution of latex beads with a mean particle size of 1µm (Sigma-Aldrich) trapped between two cover-slips.

6.2.1.6. Magnification and Horizontal field of view

The calculation of digital image magnification is dependent on a number of factors and differs to those used in optical microscopy due to the lack of eye pieces and the fact that objects are viewed on a screen. Determining the magnification of an object on a screen is dependent on knowing the PPI (pixels per inch) of the screen, the size of a single pixel on the screen, the width of the image in pixels and the actual size of the object being magnified (Figure S3). Pixel resolution is also an important factor in determining ‘useful’ magnification as, up to a point, more pixels mean that the image contains more information which will be observable to the eye when it is enlarged. Simply put, magnification is used to make viewing easier, but if information is not already in the image increased magnification will not show any more detail.

Horizontal field of view (HFOV) and full magnification were measured using a calibration slide with a 1mm marker and 10µm subdivisions. The lower magnification and HFOV were measured with a 15cm ruler with 1mm subdivisions.

6.2.2. Software

Where the previous section described the hardware components and their construction, this section will describe the software which was developed and/or used in the conjunction with the hardware to complete the system.

6.2.2.1. Image capture

The microscopes are bundled with native image capture software, however, due to the unique requirements of this system, it was necessary to write a custom application. 'AutoKams' is a standalone application designed to capture images from USB camera devices, such as web cams and microscopes (Figure 6.2). It was developed specifically for my work with the help of Junian Triajianto, an independent software coder. The software was written in Microsoft Visual Studio 2010 with C# as the programming language. The application is powered by AForge.NET framework (http://www.aforgenet.com/). In many cases when identical USB devices (such as the three microscopes) are connected to the same PC they conflict as the computer is unable to identify them uniquely, this leads to the PC crashing, or the display of only a single camera. Although some devices are able to run in tandem, this was not the case with any of the microscope models I tested. 'Autokams' was written to allow for the simultaneous capture of multiple USB devices of the same model when running on a machine with multiple USB controllers. Images for time-lapse studies were typically captured every 60,000 milliseconds for 1 hour using the capture control box and are automatically saved to labelled folders. The software is open-source, licensed under LGPL v3, and can be downloaded at: http://lab.junian.net/AutoKams. The software was tested for stability on several Windows PCs, altered accordingly, and was shown to be compatible with three different models of USB microscope (400x maximum magnification, 8 LED, 2MP interpolated CMOS). The version described here remained stable throughout all experiments.

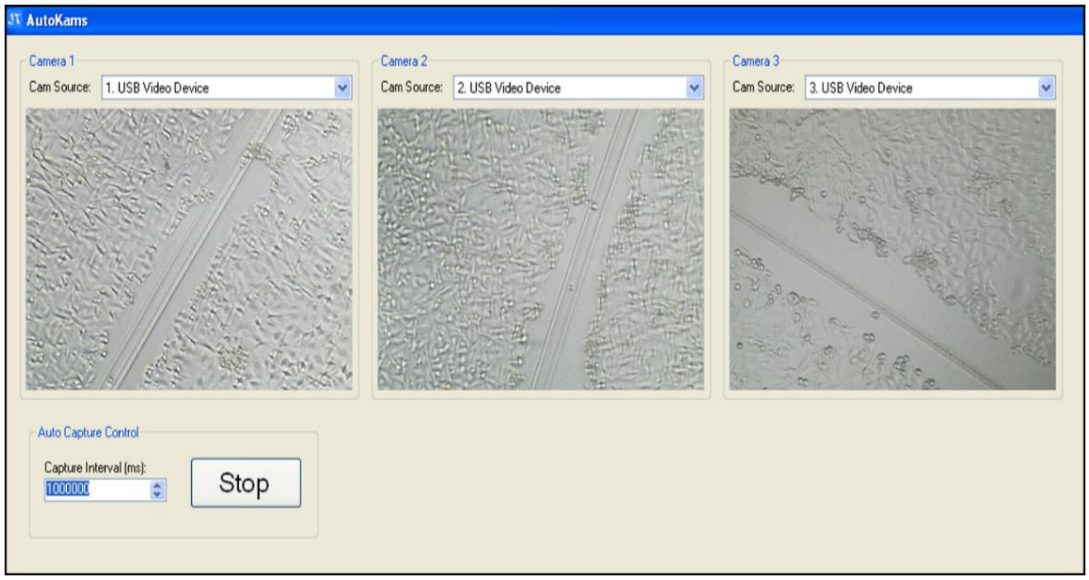


Figure 6.2. Screenshot of the Autokams software interface. *Three separate scratch assays of MDA-MB-231 cells displayed simultaneously on Autokams.*

6.2.2.2. Image preparation and analysis

Image preparation and analysis was performed within the open-source Java-based image processing program ImageJ (NIH, version 1.44). To set the precise scale an image of an object of known distance was uploaded, then, using the 'set scale' option the corresponding size in pixels could be set and applied to other objects. Experimental images (Figure 6.3A) were loaded as a stack and converted to 8-bit (Figure 6.3B). Uneven illumination often occurs in microscope images and is difficult to eliminate completely, for this reason the images were also subject to the 'subtract background' tool (Figure 6.3C). The stack was then thresholded to create a binary image with better contrast between cells and background (Figure 6.3D). The final stage of image preparation was to correct for any minor drift that may occur; this was achieved using the 'image stabilizer' plug-in. To analyse cell motility the plug-in 'MTrack2' by Nico Stuurman was used (Figure 6.3E). Minimum track length was set at 10 frames. Although there was no chemoattractant used I also analysed the same data using the ImageJ chemotaxis tool by Gerhard Trapp as an example of further applications (Figure 6.3F).

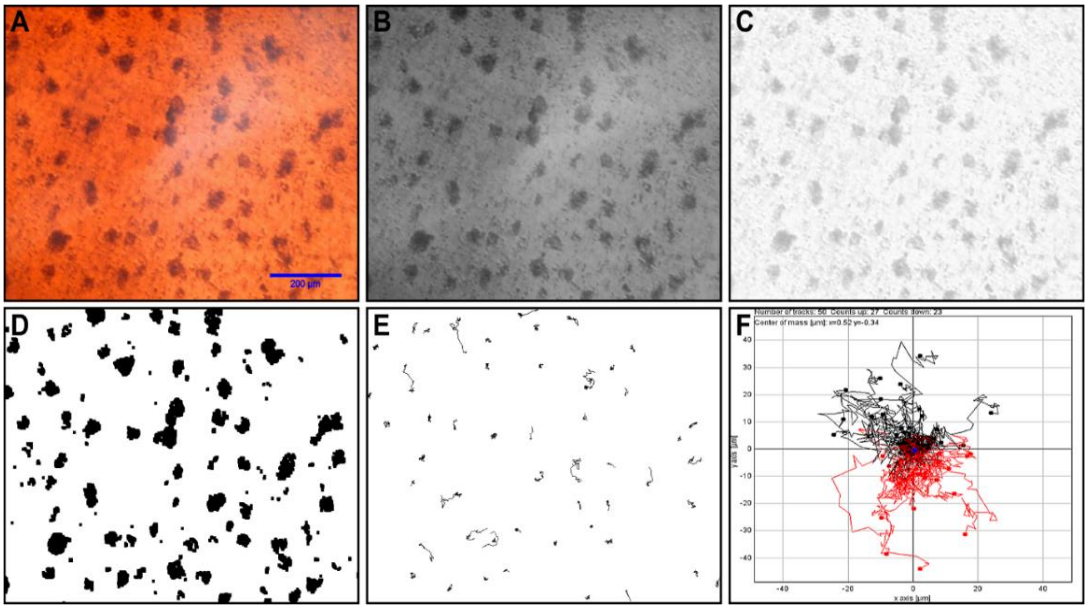


Figure 6.3. Raw image preparation and analysis process in ImageJ. *A) Original image B) 8-bit black and white C) Background subtraction D) Thresholded E) MTrack2 cell trajectories F) Chemotaxis plot.*

6.2.3. Assays

This section describes the procedure for the validation assays in which the finished system was tested and also the 'primary assay' involving the test chemicals.

6.2.3.1. Cell tracking

Cell tracking was performed on cells from three different species: primary hemocytes (immune cells) from *Biomphalaria glabrata,* mouse juvenile Sertoli cells (SC5) and human breast cancer epithelial cells (MDA-MB-231).

B.glabrata handling was as described in Chapter 2 (Section 2.2). Hemocytes were diluted 50% in CBSS and allowed to settle for 30 minutes at 27ºC before time-lapse began. *B.glabrata* hemocytes were prepared in two separate conditions; cells were either left to attach to the plate or were placed onto a surface of 0.01% poly-L-lysine according to Boehmler *et al.*, (1996) with some minor modifications.

Both the SC5 and MDA-MB-231 cells were seeded into wells of a 96-well plate in phenol red-free DMEM medium (10% fbs, 1% Glutamax and 1% penicillin-streptomycin - Sigma Aldrich) and allowed to reach approximately 20-30%

confluence. The mammalian cells were kept at 37ºC during imaging. All time-lapse images were taken every minute for 1 hour.

6.2.3.2. Scratch assay

Scratch assays are a very widely used method to investigate wound healing in various cell types. I performed scratch assays using MDA-MB-231 cells seeded into 3 wells of a 6-well plate to near full confluence using the same culture media as for cell tracking. Scratches were made in each well using the tip of a sterile scalpel. Each well was positioned over a separate microscope, and the thermostat was set to 37ºC. Images were captured every 4 minutes from each camera over a period of 10 hours.

6.2.3.3. *Artemia* development

The effectiveness of the microscope for imaging three very different samples simultaneously was tested using shrimp from the genus *Artemia*, as they are a common test organism and developmental stages are clearly distinguishable. Cysts were purchased from ZM foods (Winchester, UK) and were cultured in 20% salinity at 28ºC. Samples from different life stages were pipetted into separate wells of a 6-well plate or placed on a microscope slide and imaged at the two different magnifications.

6.2.3.4. Test chemical assays

Having developed and validated the test system, tracking assays were performed on the four test chemicals. For these assays the un-spread cell technique of Boehmler *et al.*, (1996) was chosen, as this was considered most similar to the *in vivo* response and the same approach was used for the encapsulation assay (Chapter 4). For each chemical, results were collected from 4 independent assays (cell pools) on different days.

6.2.4. Statistical analysis

Analysis was performed on the MTrack2 data generated from the time-lapse files (see published paper; Movie S1, S2, S3 and S4). As the data for comparison of cell types (Figure 6.9) failed to meet all the assumptions of an ANOVA and was not amenable to transformation a Kruskal-Wallis test was performed in SPSS version 20 (IMB) to determine whether recorded velocity values differed significantly for the various cell types. As a post-hoc test pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons. The dose-response assays for the test chemicals were performed in Graphpad Prism 6.0 using linear and non-linear regression.

6.3. Results

This section describes the results for the calibration and validation of the microscope system as well as those for which the final system was originally developed.

6.3.1. Microscope Functional Parameters

This sub-section describes the outcome of experiments to test the 'functional parameters' of the microscope system i.e. the calibration, stability and accuracy of the individual components of the system on which the reliability of the final assay results depends.

6.3.1.1. Heating stability

After approximately 1 hour the desired temperature (27^{o}C) was achieved (Figure 6.4). After the first hour the temperature did not vary by more than 0.5^{o}C either side of this temperature for the remaining 19 hours although a gradual increase could be seen.

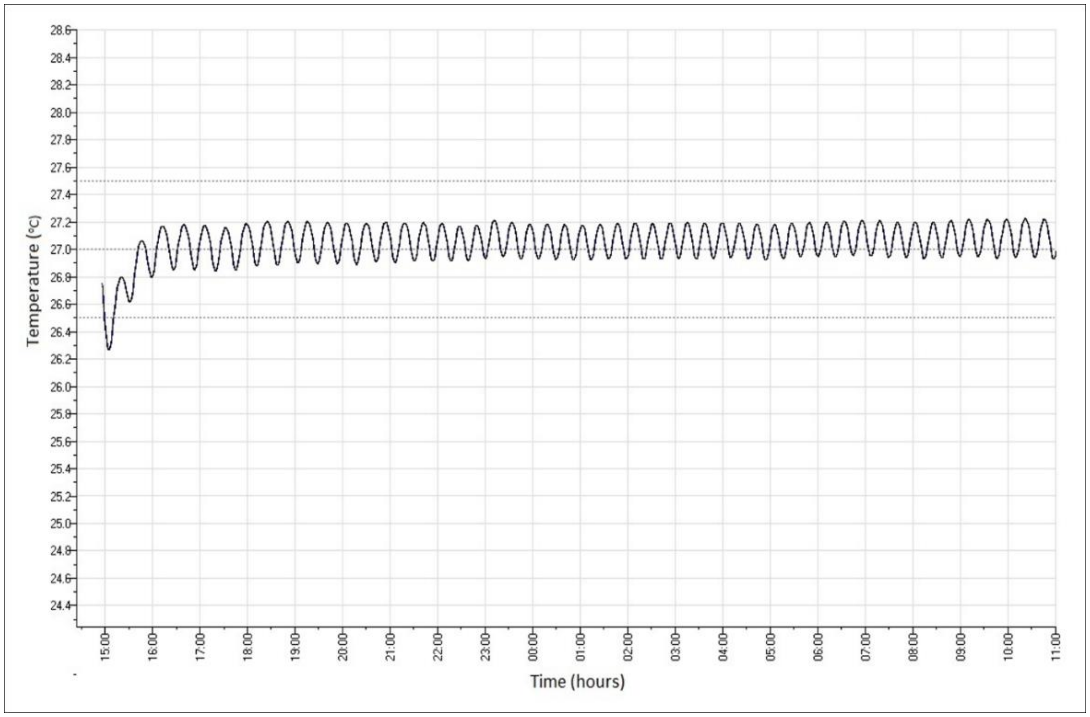


Figure 6.4. Incubator temperature stability over time. *Incubator temperature readings (°C) taken every minute for 20 hours.*

6.3.1.2. Field of view and magnification

Based on the calibration slide, the horizontal field of view at maximum magnification was determined to be 0.99mm (Figure 6.5 A) and 13mm for the lower magnification (Figure 6.5 C) at 640x480.

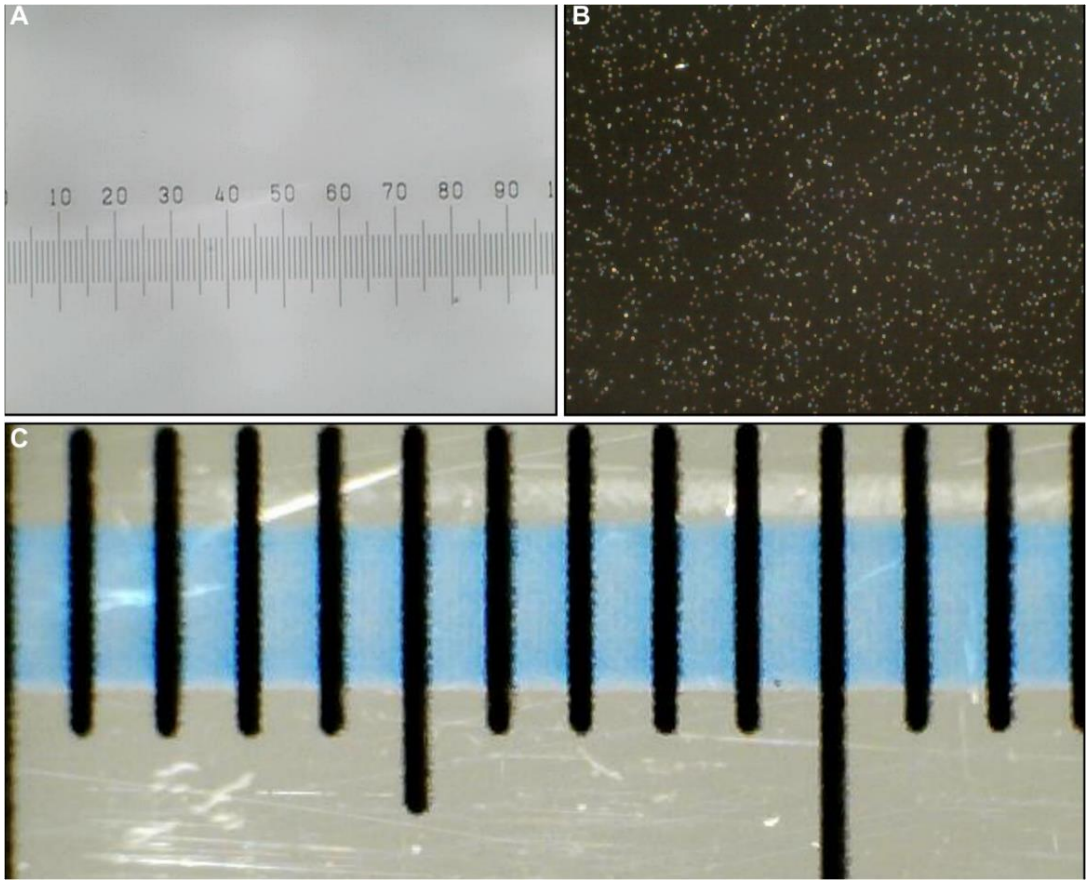


Figure 6.5. Images used for calibration of the microscopes. *A) 1 mm graticule with 10 mm subdivisions at 206.8x magnification B) 1 mm latex beads at 206.8x magnification C) 15 mm ruler at 15.76x magnification with 1 mm subdivisions.*

I estimated the maximum magnification at the pixel resolution chosen for cell tracking (640x480) to be 206.8x on a screen with a PPI of 78 and the lower magnification to be 15.7x. This calculation appears accurate when the image taken with my microscope is enlarged by 149% (the percentage difference in size between 206.8x and an image taken at the same pixel resolution on a conventional inverted microscope at 310x; Figure 6.6 A and B). The microscopes are capable of greater magnification if pixel resolution is set to the maximum of 1280x960.

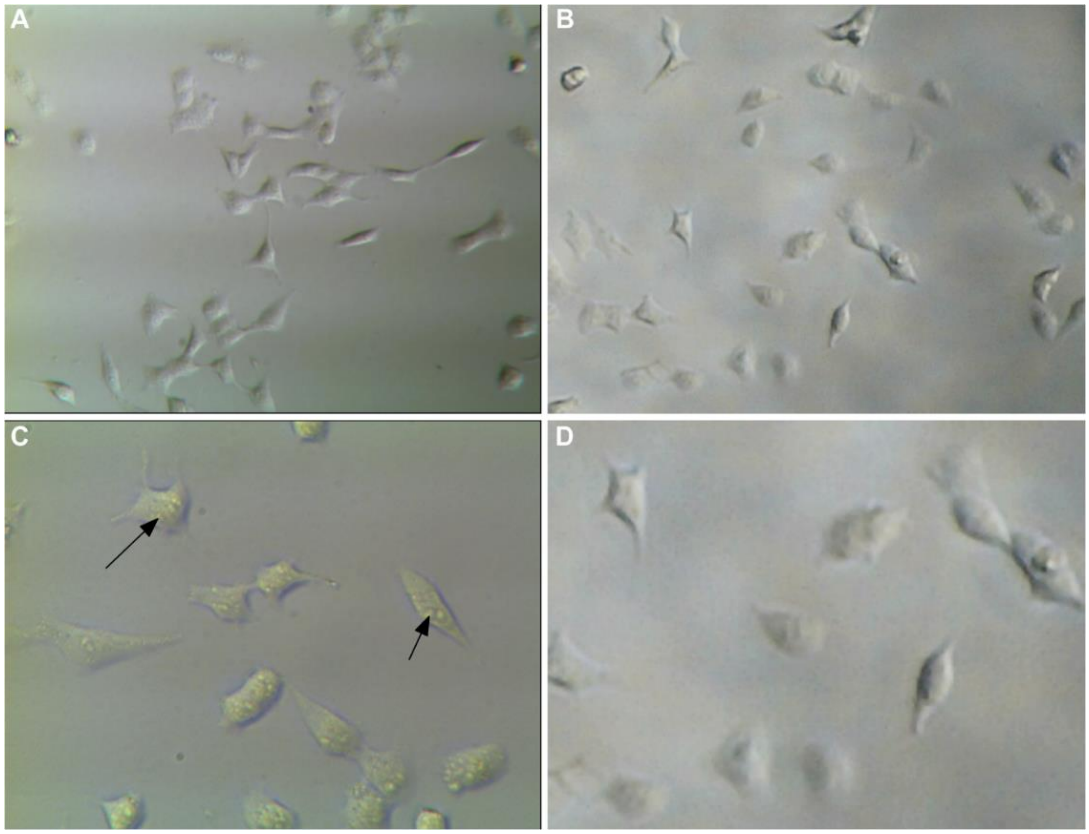


Figure 6.6. Comparison of MDA-MB-231 cell images between my system and a conventional optical microscope. *A) 310x image taken at 640x480 with a conventional inverted microscope B) 206.8x image taken at 640x480 with my system and enlarged post acquisition by 149% to match the size C) 620x image taken at 1280x960 with a conventional inverted microscope, arrows show intra-cellular detail D) 1280x960 image taken with my system at full magnification (413.6x) and enlarged post-acquisition by 149% to match the size.*

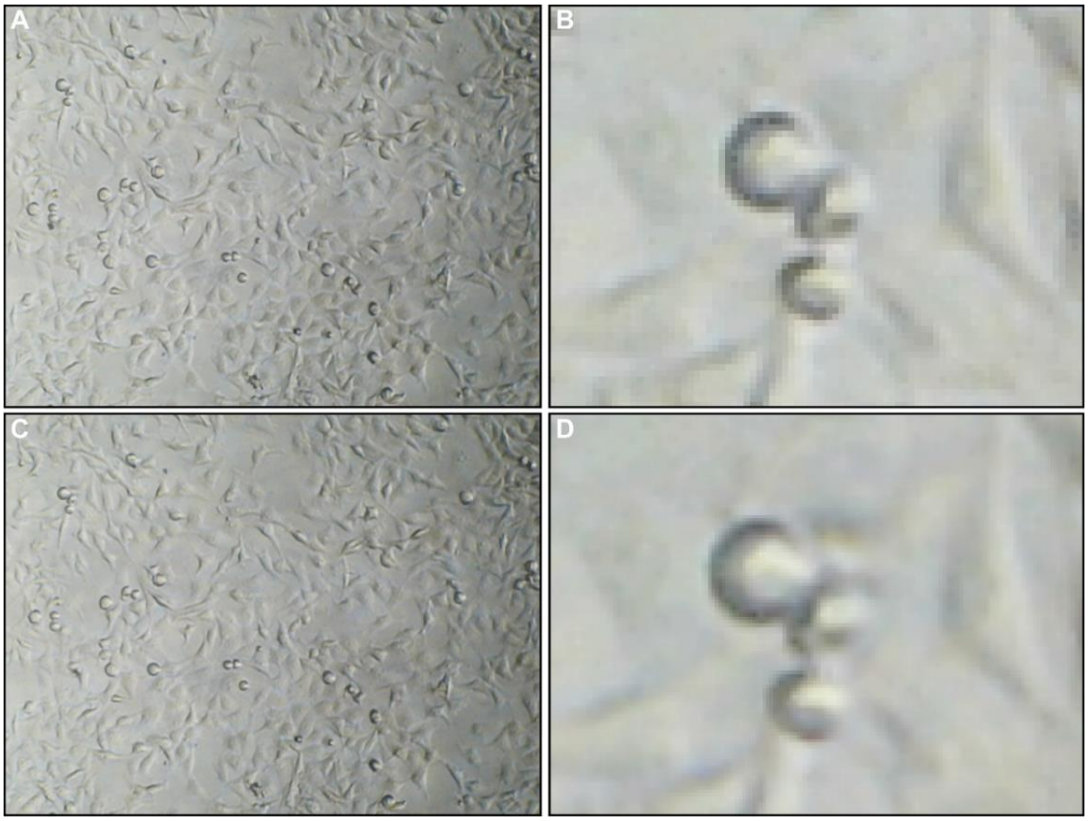


Figure 6.7. Comparison of digital microscope pixel resolutions between 640x480 and 1280x960. *A) 640x480 image at full magnification B) 640x480 image enlarged post-acquisition to 800% C) 1280x960 image at full magnification reduced in size by 50% D)1280x960 image enlarged post-acquisition to 400%.*

6.3.1.3. Pixel resolution

The improvement in image quality when increasing pixel resolution from 640x480 to 1280x960 was found to be minimal at the magnification used for cell tracking (Figures 6.7; A and C) but doubles the file size (and therefore storage requirements). The impact only becomes apparent when the images are increased significantly in size post-acquisition, a noticeable increase in pixilation can be seen when enlarging an image taken at 640x480 by 800%, which is the same on-screen size as an image taken at 1280x960 enlarged by 400% (Figures 6.7; B and D), however these sizes are far in excess of what would be useful for my purposes.

6.3.1.4. Spatial resolution

At full magnification, 10µm divisions were clearly visible and 1µm beads were distinguishable. However, the measured diameter of the beads was larger than 1µm suggesting that the microscope was picking up the reflected light (Figure 6.5; B). Spatial resolution was therefore determined to be at least 10µm. Crucially I was able to clearly resolve all cell types tested (size ranges 24-58µm) and could distinguish individual cells from one another and from the background.

6.3.1.5. Comparison with a conventional microscope

The imaging capability of my system was compared to a conventional inverted microscope (GX XDS-3) fitted with a 1.3 mega-pixel camera. The highest magnification on the conventional microscope (620x) was greater than my system (413.6x), maximum pixel resolution of images was the same (1280x960). Spatial resolution on the conventional microscope was higher and intra-cellular detail could be seen at the highest magnification that could not be distinguished in my system when images were enlarged to match the size (Figure 6.6; C and D). Images can be enlarged post-acquisition, or by using interpolation, to increase on-screen size but this results in 'empty magnification' and it resolves no more detail than the original image while resulting in substantial loss of quality (Figure 6.7; A and B). When comparing the two systems using lower magnification and pixel resolution (as was used for cell tracking assays) there appears little difference in the quality of the images and both would be equally suitable for motility work (Figure 6.6; A and B). Finally, the cost of assembling my system was approximately £161, whereas a conventional inverted microscope (with camera) retails for approximately £3,250.

6.3.1.6. Lighting

In order to achieve optimum resolution and improve signal-to-noise ratio (SNR) the in-built LED lights were turned off. It was found that for most cell samples optimum SNR was achieved from an external overhead light source (Figure 6.8).

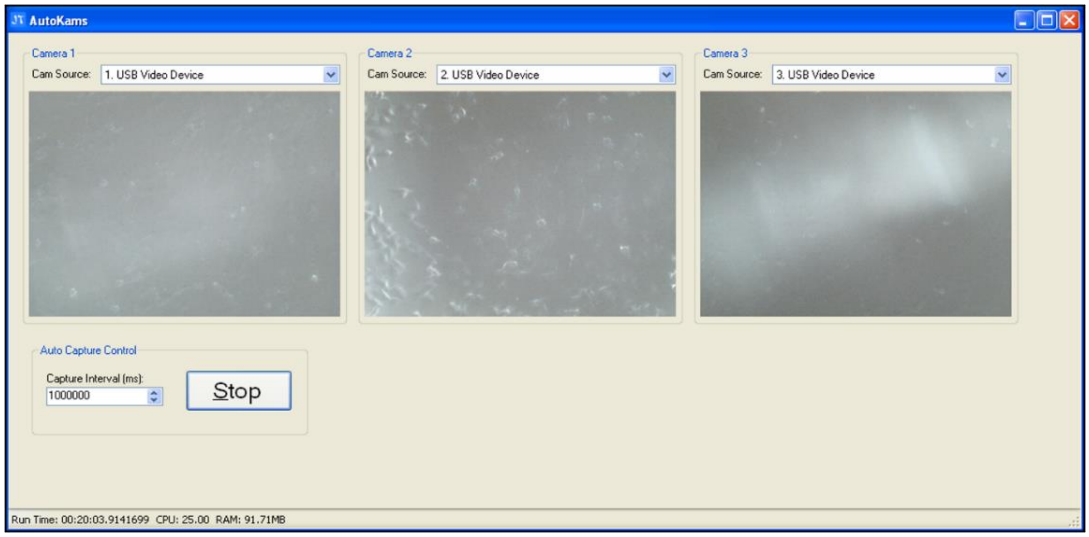


Figure 6.8. Screenshot of the Autokams interface showing the result of illumination from the in-built LEDs alone.

6.3.2. Assays and applications

This section describes the results of the validation and test chemical assays in which the microscope system, having been satisfactorily calibrated in the previous section, was employed. Also described is an experiment designed to highlight wider capabilities and applications of the system.

6.3.2.1. Cell velocity

According to visual inspection, the Kruskal-Wallis test distributions of velocity scores were not similar for all groups, and velocity values differed significantly between cell types, χ^2 (3) = 106.531, p = >.001. Pairwise comparisons showed significantly lower velocity of spread *B.glabrata* cells (0.81 ± 0.01 µm/min; Figure 6.9) compared to all other cell types, including *B.glabrata* on PLL (2.21 ± 0.01 µm/min; Figure 6.9). There were significant differences in velocity between MDA-MB-231 cells (1.17 ± 0.004 µm/min; Figure 6.9) and both *Biomphalaria* cell culture methods, but not to SC5 (1.24 ± 0.006 µm/min; Figure 6.9). Likewise, the velocity of SC5 cells differed significantly from both *Biomphlaria* cell culture methods but not from MDA-MB-231 cells (Figure 6.9). Assumptions of the ANOVA test were

most likely not met since I chose to accept results from cells of differing track length (between 10-60
tracks).

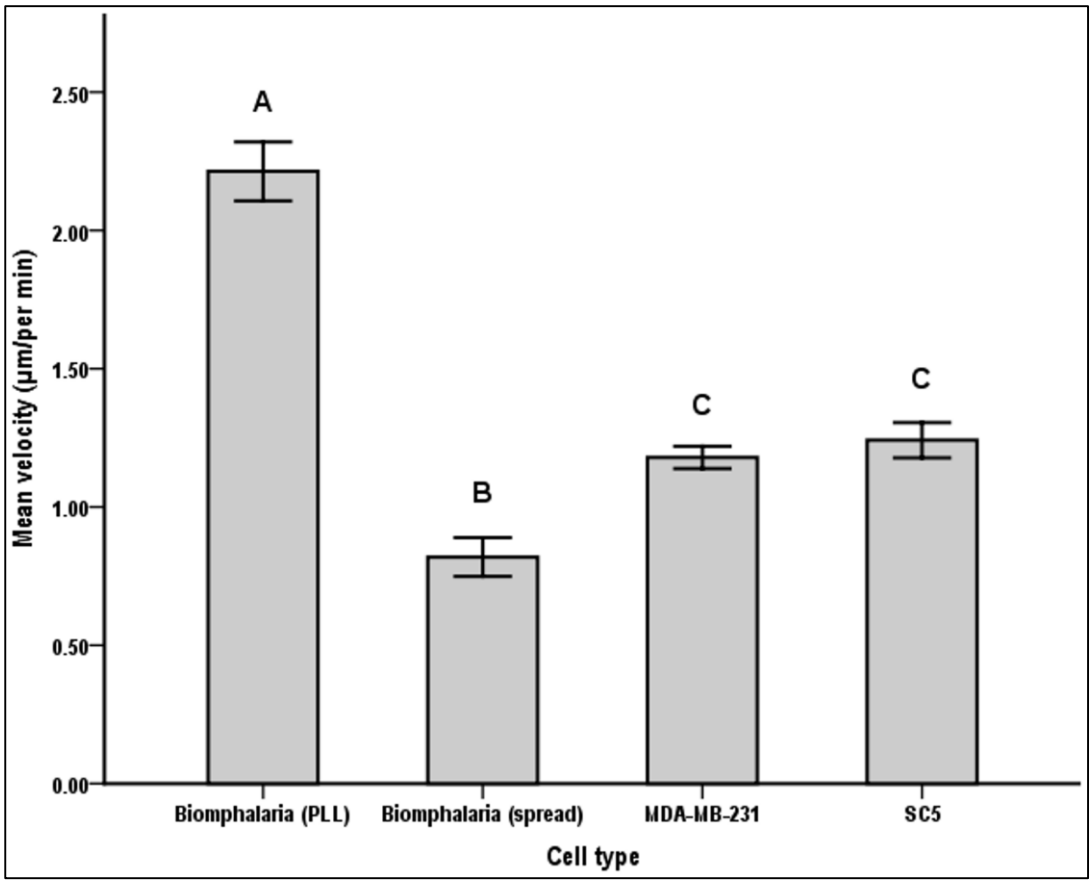


Figure 6.9. Mean velocity (± SE) in µm/min of the 4 cell types. Shared letters indicate no significant difference between cell types according to K-W test pairwise comparisons.

6.3.2.2. Scratch Assay

The system was tested for use in performing scratch assays. Not only were the microscopes able to detect cells and scratches sufficient for use in the assay, but it was also found to be relatively simple to align three separate scratches/wells simultaneously (Figure 6.10). More difficult was keeping each scratch oriented in the same way, but this has no impact on the validity of the assay and can easily be altered post-acquisition.

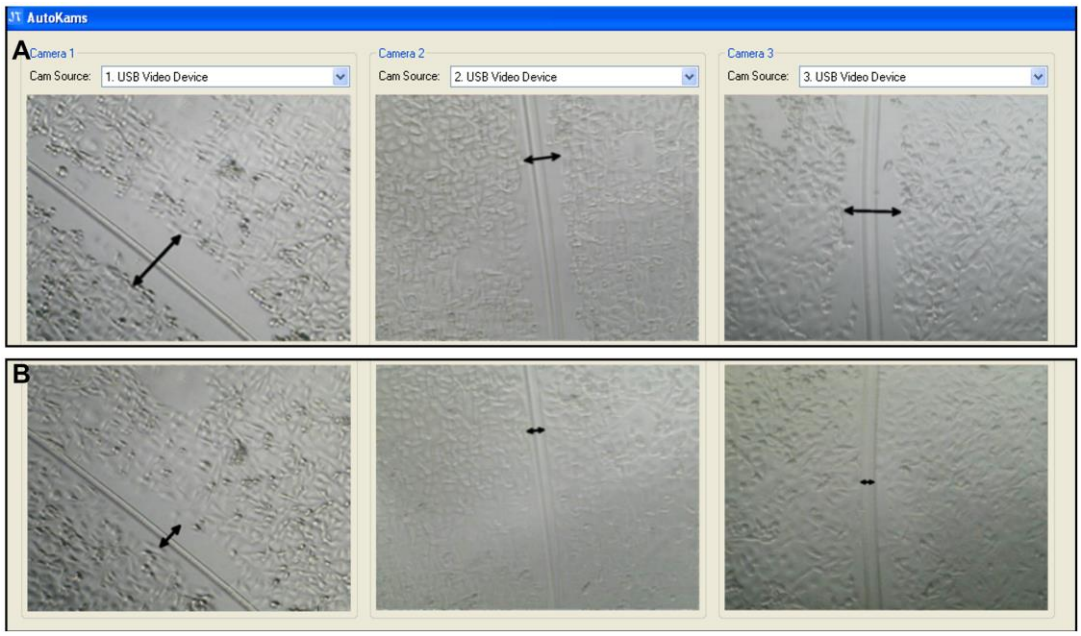


Figure 6.10. Screenshot of Autokams interface showing individual scratch assays simultaneously recorded on each camera. *A) cells at 0 hr B) cells after 10 hrs; Arrows show the scratch channels.*

6.3.2.3 *Artemia* development

I was able to demonstrate the ability of the system to simultaneously image three distinct specimens (Figure 6.11). Furthermore, it was possible to independently alter the magnification of a chosen sample separately. In the case of *Artemia* development, the smaller life stages (cyst, nauplii) can be viewed in detail by higher magnification while leaving the much larger adult stage at a lower magnification (Figure 6.11; B).

Figure 6.11. Screenshot of Autokams which demonstrates the ability of the microscope system to image distinct samples in different manners simultaneously. *A) Cyst, nauplii and adult Artemia at 15.76x magnification B) Cyst and nauplii at 206.86x, adult at 15.76x C) All three stages at 206.86x magnification.*

6.3.2.4. Study chemical testing

With the exception of E2 (which was best modelled by a 2nd order polynomial), all chemicals followed a negative linear relationship (Figure 6.12). All relationships were relatively strong and suggested that the majority of variation in cell velocity was explainable by chemical dose. Effluent displayed the strongest relationship (Y = -0.6705*X + 2.359) with a very high r^2 value of 0.9754 followed by DDE (r^2 = 0.8483, Y = -0.1046*X + 2.290) and E2 (Adjusted R^2 of 0.8155). The relationship with BPA was somewhat weaker, but exposure still explained a considerable degree of the variation in cell velocity (r^2 = 0.5122, Y = -0.1749*X + 2.572). BPA appeared to induce a degree of stimulation, relative to control, at the lowest dose but overall

followed a negative linear relationship with increasing dose. In each case, diagnostic tests were satisfactory (i.e. normality, insignificant deviations from linearity in the case of BPA, effluent and DDE).

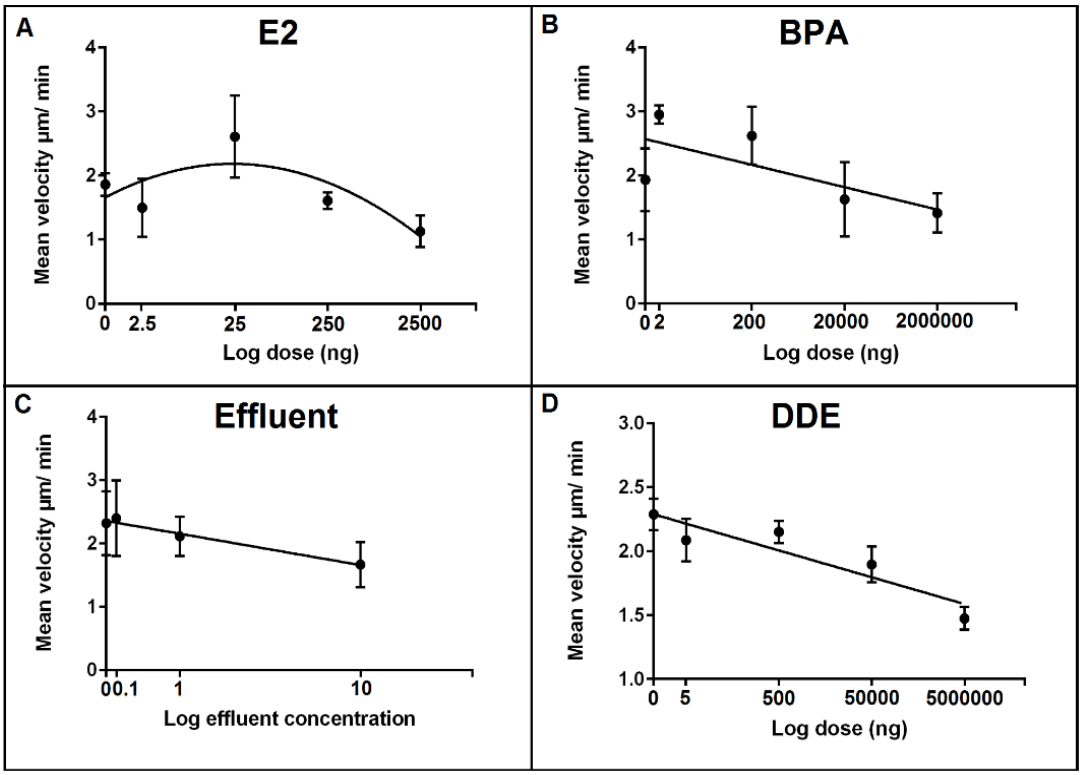


Figure 6.12. Regression relationships between log dose (X axis) of test chemicals against *B. glabrata* hemocyte motility *in vitro* (Y). *Data points for hemocyte motility plotted as mean velocity (µm/min)* ± *SEM of 3 independent replicates. A) Hemocyte motility response to E2 exposure B) Hemocyte motility response to BPA exposure C) Hemocyte motility response to Effluent exposure D) Hemocyte motility response to DDE exposure.*

6.4. Discussion

This study began solely as a means to investigate motility of *B.glabrata* hemocytes in the presence of xenobiotics, due to a lack of existing methods that were time and cost-effective, within the scope of the project as a whole. However, I soon realized its potential for broader applications and the work eventually centred around the development of the system.

Here I demonstrate the development and application of a low-cost microscope system for multiple time-lapse imaging as well as its application toward my project hypothesis. By inverting commercially available digital microscopes and employing an external light source the stability, working distance and signal-to-noise ratio are

improved considerably, and images were comparable to a much more expensive standard inverted microscope/camera system. I also show that the system can support a number of microscopes simultaneously, hence increasing the throughput capability of time-lapse studies captured onto a single PC. Parallel cell tracking and scratch assays were carried out on independent samples with relative simplicity, and the ability of the system to image samples of different sizes simultaneously is demonstrated. There was very little difference in the images taken using my system (at maximum magnification with a pixel resolution of 640x480) compared to a conventional inverted microscope at a similar magnification (Figure 6.6; A and B). Differences in resolution between the two types of microscope only become apparent when attempting to use higher magnifications (Figure 6.6; C and D). To achieve the highest useful magnification of the digital microscope (413.6x) the pixel resolution can be set to 1280x960. The maximum magnification of the commercial microscope was 620x (although higher objective lenses can be purchased). Figure 6.6; C shows an image taken on the conventional microscope at its maximum magnification. As the maximum magnification is higher on the conventional microscope I needed to enlarge my images post-acquisition in order to make a comparison at the same size. Since the limits of the lens and sensor have already been reached for my system this kind of post-acquisition manipulation is an example of empty magnification (Figure 6.6; D). There is considerably greater detail in the image taken with the conventional microscope due to its greater spatial resolution. Clearly my system would not be suitable for investigating intracellular events, but is perfectly adequate, even at a lower pixel resolution (640x480), for cell tracking. Indeed, greater magnification is not required for this purpose, especially when factoring the increased cost (Yarrow *et al*., 2004).

A number of published studies now demonstrate the rapidly improving capabilities of digital imaging sensors compared to the existing standards in microscopy or explore their potential applications (Tseng *et al*., 2010; Zimic *et al*., 2010; Smith *et al*., 2011, Linder *et al*., 2013). Despite this, one of the main limitations to many of the lower-cost time-lapse systems is the rate of data collection or 'throughput'. Here I demonstrate the impressive capability of CMOS sensors to image cells in tandem as an alternative to the expensive systems on a cost-performance basis. As the sensors used are small and light weight, it is possible to decrease the size and cost of my system substantially by sourcing sensors directly from the manufacturer (rather than buying a

commercial system sold as a microscope) as these can cost as little as $1.50 a unit (Zheng *et al*., 2010). Stripped back sensors would allow more microscopes to fit into a smaller space (for example in a 3x3 grid pattern) and at much lower-cost. The actual number of microscopes that could be run simultaneously on one PC would depend on the processor speed, memory (RAM/cache) and the number of available USB ports/controllers. However, given the low frame rate required for time-lapse studies it is likely that a larger number could be supported. Indeed, by sourcing components directly, I believe that the three camera system described here could be reproduced for as little as 20 GBP. The ability to run three identical microscopes in tandem actually proved to be one of the biggest challenges in developing this system. The microscopes' native software did not support multiple models and I was unable to find any third-party software that met the criteria. Therefore, a new software platform was written specifically for my requirements, demonstrating the importance of collaboration between scientists and programmers; a point that has been stressed by others (Meijering *et al*., 2009; Cardona and Tomancak *et al*., 2012). I hope that the open-source nature of the software will allow it to be more widely adopted and adapted for use with other platforms and in communities such as ImageJ and Micro manager.

When performing cell tracking or wound healing assays it is common to render the images post-acquisition to enhance the signal (cells) to noise (background and artefact) ratio (SNR), especially when using automated analysis programs. This demonstrates that, beyond a certain point, the overall resolution of the original image has little bearing on the quality of the data (Frigault *et al*., 2009). In many cases, it may be unnecessary to invest significant sums of money for high resolution images or high magnification when the same data can be obtained using a less expensive microscope. In the case of cell tracking, high magnification results in a reduced field of view and, consequently, lower sample size and statistical power. Obviously there are conditions where it is crucial to obtain as much information from the image as possible and there is also a minimum level needed to perform tracking assays, especially for slow moving objects. However, in situations where robust motility data is prized above all (attractive images being a secondary consideration) there may be little merit in investing large sums of money.

Four different cell types were used to test the ability of the system to measure cell velocity. While *B.glabrata* hemocytes were the main species of interest in this study I

decided to test how applicable it would be to more widely used cell types. *B. glabrata* haemocyte cells moved at a velocity of 0.81µm/min ± 0.01 on an untreated surface, compared to 2.21µm/min ± 0.01 on a poly-L-lysine treated surface (Figure 6.9). This is consistent with previous reports of an increase in velocity from 0.99µm/min ± 0.72 (untreated) to 5.13µm/min ± 2.02 on poly-L-lysine coated surfaces using *B. glabrata* haemocytes (Boehmler *et al*., 1996), albeit from a different strain. For SC5 cells an average velocity of 1.24µm/min ± 0.01 was recorded but I was unable to find sufficient data in the literature for comparison. For MDA-MB-231 cells I found an average cell velocity of 1.17µm/min ± 0.004. There are several existing studies which have investigated motility in MDA-MB-231 cells which vary from 0.4-2.5µm/min (Wang *et al*., 2004; Onuki-Nagasaki *et al*., 2008; Irimia and Toner, 2009; Mak *et al*., 2011; Zheng *et al*., 2013), likely due to the variation in experimental techniques. My results are in the middle of this reported range, demonstrating that this system is capable of generating quantitative data comparable to those reported in other studies using more expensive equipment.

To my knowledge only one other study exists which has looked at the motility of molluscan hemocytes in relation to xenobiotics. Rioult *et al*., (2014) measured the *in vitro* motility of mussel hemocytes collected at polluted and non-polluted sites. They found that mussels collected from the polluted site displayed significantly reduced motility when compared to those from the clean site, giving the first evidence of alterations in hemocyte motility in xenobiotic-exposed molluscs. Direct comparison is not possible due to the difference in study designs (in the present study hemocytes were exposed *in vitro*) but we have also shown considerable reductions in motility with increasing doses of our test chemicals (Figure 6.12). One exception to this was for E2, which showed a non-monotonic response in the middle of the dose range. In terms of mechanisms for the observed response, as with phagocytosis (Chapter 5; Section 5.4) the ERK signalling pathway would again appear to represent a potential candidate since it is key in regulating cell motility and has also been shown to be negatively impacted in mollusc hemocytes by *in vitro* exposure to environmentally relevant doses of xenobiotics, including E2 and BPA (Canesi *et al*., 2004; Porte *et al*., 2006; Zelck *et al*., 2007).

While the system I have described cannot match the impressive capabilities of the high-end devices needed for large throughput tasks, such as drug discovery, I believe

that my system would appeal to researchers with limited budgets. In these circumstances the system could be applied to life science education including demonstrations of different stages of development and the effect of exposure conditions in various macro and microscopic organisms (Figure 6.11). This system produces results comparable to standard inverted microscopes, whilst generating three times the data in a single run. The low memory demands of the system mean that it is likely to be compatible with older machines; for example, I was able to run experiments successfully using a 10-year-old PC (IBM ThinkCentre 9210). The system is also portable, enabling experiments to be carried out directly in the field.

There are still many areas in which the system could be improved given more time. While the current manual focusing system works well I believe that this process could be automated by the software in order to reduce the likelihood of accidentally moving the field of view while touching the microscope. An important feature which the system currently lacks, but is commonly required for cell culture, is a CO_2 pump. The lack of a CO_2 pump is not believed to have affected the cell tracking experiments shown here, as the DMEM media contains CO_2, which would not have depleted in the course of 1 hour (Coutu and Schroeder, 2013). The absence of a CO_2 pump may, however, affect longer-term experiments (such as the scratch assay), and this could be addressed by placing the entire system inside a CO_2 incubator or finding ways to miniaturize the CO_2 supply system. One such design, with the inclusion of a miniaturized CO_2 delivery system, is described by Chung-Liang *et al.,* (2005).

In conclusion, I demonstrate the ability of a novel cell tracking system to perform multiple simultaneous time-lapse studies on various cell types. Due to its low-cost, portability and commercially available components I believe that this system has the potential to enable time-lapse studies by non-specialist departments and schools, and may be a practical solution for researchers with limited financial resources. Since this project was first made public in August 2014 it has received a significant amount of media exposure in several countries and as a result I am pleased to have been contacted by numerous researchers in the fields of cancer, neurobiology and geology. Furthermore, one of these systems has been taken to Tanzania for field work use by the Natural History Museum, this is something which could potentially see it being

applied to schistosomiasis research within transmission countries which are in need of low-cost alternatives to commercially available scientific equipment.

Chapter 7: *In vivo* Effluent Exposure Study

7.1. Introduction

In vitro testing is an integral part of the biological investigation process, in which its advantages are most pronounced when considering scale, statistical variability and time.

Since *in vitro* tests typically involve small organisms and/or small parts of larger organisms (i.e. cells) the number of animals used, space, volumes of reagents required and waste produced are all considerably lower than *in vivo* (whole animal) tests (Baksi and Frazier, 1990; Ankley, 2008). The smaller scale means that more samples can be screened (and more quickly) which yields faster and more numerous results. Screening multiple samples, as well as the ability to include internal controls and stabilise the environment, means that variability is typically considerably lower for *in vitro* tests (Mothersill and Austin, 2011).

Results from *in vitro* tests are often used to extrapolate and form conclusions at larger levels of biological organisation, which is both a great strength and key disadvantage of *in vitro* tests (Mothersill and Austin, 2011).

It is clear that no matter how carefully or how faithfully represented *in vitro* conditions are, they provide us with only potential mechanistic explanations of processes that occur in the higher animal (in the absence of whole animal pharmacology and pharmacokinetics) and are therefore best used to guide and inform subsequent investigation on whole animals. The limitations of *in vitro* assays can be highlighted using various examples, but ultimately all stem from the problem of reductionism.

When cells are collected for *in vitro* use they are first removed from the three-dimensional geometry in which they naturally exist and are then usually propagated into a two-dimensional environment (Freshney, 2000). While methods in three-dimensional culture exist, they are far from the standard approach and even these are not truly representative of the *in vivo* process as they require various treatments before use (Haycock, 2011). When 3D cell cultures are used they still rarely consist of primary cells and instead involve the use of established cell lines which are often cultured over decades until they eventually represent markedly different phenotypes and lose many properties of the progenitor (Mothersill and Austin, 2011). When cells are removed from the environment where they naturally form part of a larger

system they can lose their ability to interact with other cell types and often are no longer under the overall control of the complex factors which form the endocrine or nervous system (Mothersill and Austin, 2011). The ability of cells to interact and form organs also alters the concentration and physical property of the xenobiotic chemical itself. For example, liver metabolism and excretion may rapidly eliminate compounds and can also transform them into more or less active metabolites and these may target the same or different cell types and processes within the intact animal. Not all cell types within an animal will be equally effected by a given compound and the overall toxicity will be disproportionately contributed to by certain cell-types. Experiments in fish, for example, have shown that the toxic impact of metals is primarily as a result of accumulation in gill tissue and the associated damage this causes. Consequently, the impact of metals on other fish cell types in an *in vitro* system would likely underestimate the potential harm to the whole animal (Mothersill and Austin, 2011).

Given the numerous different ways in which a compound can change and interact with cells in an *in vivo* system it is often the case that the absolute sensitivity in whole animals is greater than in cell cultures (Segner and Lenz, 1993; Kilemade *et al*., 2002). This is particularly likely if an animal is developmentally exposed i.e. as it grows and matures, since compounds would have the potential to disrupt physiological processes as they are forming, something which can result in more profound outcomes than if development is already complete.

Since the ultimate goal of *in vivo* testing in ecotoxicology is to achieve a greater degree of environmental relevance it seems equally desirable that not only the animal but also the test compounds are of a 'real-world' origin.

Historically the majority of ecotoxicology studies have focused on responses of cells or tissues of intact organisms to various doses of a single chemical. However, in the environment many different compounds exist together in what are referred to as 'mixtures' (Gagnaire *et al.*, 2006). Even when individual concentrations of similarly acting xenobiotics in natural waters are very low (sub ng/L) the combined concentrations from the mixture can prove toxicologically significant with respect to aquatic organisms (Brian *et al.*, 2004). As a result, the field of mixture-toxicology emerged in order to provide a more environmentally relevant model for laboratory tests.

Municipal effluents are recognized as a major source of many environmental contaminants, including polyaromatic hydrocarbons, pesticides, surfactants, steroids, and metals (Gagné *et al*., 2006). Effluents are known to contain many chemicals with different mechanisms of action which can result in many different effects on an exposed organism. Therefore, while many mixture studies will combine several different chemicals in the laboratory, exposure to real sewage effluent is the gold-standard in environmental relevance as it represents a 'real-world' mixture (Ankley, 2008).

7.1.1. Aims

The aim of this study was to broaden and strengthen the testing of the central hypothesis, that pollution affects the parasite-host relationship, by including a more environmentally relevant *in vivo* approach to supplement the previous *in vitro* work.

I investigated the *in vivo* parasite-host interaction in the presence and absence of different concentrations of an effluent extract in order to simulate, within a controlled environment, the chemical composition of polluted urban environments where *B.glabrata* and *S.mansoni* might naturally reside.

Whereas previous chapters have been concerned with understanding if/how certain chemicals elicit an effect on key aspects of the snail immune response in isolation (*in vitro*), this chapter proceeds to investigate whether it is possible to relate such effects on these immune parameters to alterations in the parasite-host relationship *in vivo*, after snails have been developmentally exposed. Such alterations might include differences in snail infection rates, disease progression in the snail following infection with miracidia, and transmission potential (cercarial shedding).

This study also provided an opportunity for *in vitro* methods developed/used in previous chapters to be applied to cell samples taken from snails which had been exposed *in vivo.* In previous chapters' primary cells were taken from snails unexposed to chemicals or parasites and then incubated in the presence of chemicals *in vitro.* The fundamental difference between the *in vitro* tests used in this chapter and the same tests used in previous chapters is that the cells need not be incubated in the presence of chemicals since this has already occurred when they were inside the animal.

7.2. Materials and Methods

In this section I describe the materials and methods specific to the *in vivo* study. As with the other experimental chapters, what are referred to as the 'general' methods (those that are used throughout the thesis) are contained in Chapter 2.

7.2.1. Exposure Methods

When exposing whole animals to a test chemical there are four main systems which are used: static, static-renewal, recirculation and flow-through (Landis *et al*., 2011). With static systems, the water is not changed for the duration of the test, the main advantage here being simplicity i.e. the amount of chemical required is relatively small as is the amount of effort and also the amount of waste for disposal. The main limitation with this type of assay is that for longer exposure it allows considerable build-up of waste products that promote bacterial growth which may, in turn, degrade the test compounds (Landis *et al*., 2011). Rapid bacterial growth is also associated with greater oxygen depletion, although this can be offset with the addition of an air pump.

Static renewal involves exposing the test animal to the required chemical in static waster (as above), but this is partially or fully renewed at certain intervals during the study. The advantage of this system is that it effectively lessens the negative consequences of static exposure by refreshing the test chemical and removing harmful waste and bacteria. The downside with this method is that it is typically the most labour intensive as it is hard to automate and also requires more of the test chemical than a standard static system.

Recirculation systems involve using the same exposure water throughout the study, but it is effectively cleaned by passing it through a filter before returning it to the exposure tank and re-exposing the organisms to it again. While this system is automated and can maintain the quality of the water, a major shortcoming is the potential effect of filtering on the maintenance of correct test chemical concentrations in the water (Landis *et al*., 2011).

The final system is 'flow-through' which is the preferred approach, but also the most complex and expensive to setup and maintain. In these systems fresh water is continually passed through the exposure vessels (tanks) from a header tank and out

into waste, ensuring a continually renewed supply. Test chemicals are typically added continually *via* peristaltic pump at rates calculated to maintain a consistent exposure concentration (Walker, 2012).

Static-type systems are usually employed when the test material is difficult to obtain in large quantities and/or stored easily e.g. effluent samples. Static methods are also commonly used when test organisms are small in size, since the cost and effort involved in using flow-through systems is rarely justified in species which require much less space (Hoffman, 2003; Lammer *et al*., 2009). If space/expense is a significant limiting factor then static methods are preferred, but attempts should be made to compensate for some of the disadvantages by using a static-renewal approach where possible.

The majority of studies on snail species use static or static-renewal tests as snails are generally well adapted to low oxygen environments in ways that fish are not. In this study I report the findings of a developmental exposure of *B.glabrata* (from newly laid egg masses to adults) to effluent extract in a static-renewal system followed by subsequent infection of some snails with *S. mansoni*.

7.2.2. Pilot Study to Determine Optimal Exposure Conditions

A pilot study was performed in order to assess the optimal density and water-change parameters for the snails. Since I decided to use an effluent extract in the *in vivo* exposure, the volume of available effluent that could be feasibly extracted in order to conduct the exposure study needed to be determined. Table 7.1 shows calculations for the volume of effluent required in order to prepare stock doses given certain values for the doses, number of replicates, exposure length and the frequency of water renewal. Alterations to any of these parameters have a significant impact on the final volume of effluent required to extract due primarily to the highest, 10x, dose (which requires 10 times the volume of original effluent). For example, if the values for Table 7.1 were altered to account for changing the water daily (rather than every third day) the final volume of final effluent needed would increase from 99 litres to 298 litres. I therefore needed to determine the optimum exposure conditions within a feasible range of effluent volume.

Table 7.1. Example of the calculations for the total volume of final effluent required in order to obtain the desired volume of extract for use in the *in vivo* study.

Dose of effluent	**Replicate no.**	**Vol (L)**	**Exposure duration (d)**	**Proportion of water changed per day**	**Effluent required (L)**
0.1 x	6	0.16	28	0.333	0.895104
1 x	6	0.16	28	0.333	8.95104
10 x	6	0.16	28	0.333	89.5104
				Total volume of effluent Needed (L):	**99.356544**

For the pilot study, snails were kept in freshwater throughout. Individual egg masses were hatched together in the same container and then 5 juvenile snails were transferred to each of 27 glass beakers (3 beakers per treatment) to begin the experiment. The groups were maintained identically, with the exception of the density (by adjusting the water volume) and water change frequency (Table 7.2). Results were taken after an exposure length of 21 then 28 days. 21 days was chosen as the minimum time, since in the final study adult snails would be required and 28 days was chosen as the upper limit to yield snails larger but which would still be within optimum susceptiblity to infection.

Table 7.3 lists studies from the literature that were used as a reference point to inform certain decisions prior to the pilot study.

Table 7.2. Experimental groups for the pilot study based on density and water change frequency. *(n = 3 replicate beakers for each condition)*

		Density		
		1 per 100ml (5 in 500ml)	3 per 100ml (5 in 166ml)	5 per 100ml
Water change frequency	Daily (1 per day)	n=3	n=3	n=3
	2nd day (0.5 per day)	n= 3	n=3	n=3
	3rd day (0.333 per day)	n=3	n=3	n=3

A constant temperature of 27ºC was maintained by immersing the glass beakers into thermostatically controlled water trays (Figure 7.1).

Snails were fed equal amounts of food (replaced daily) and any waste food and faeces was removed by aspiration using a p20 pipette tip attached to a 5ml glass pipette tip (to ensure young snails would not pass through) and a vacuum aspirator.

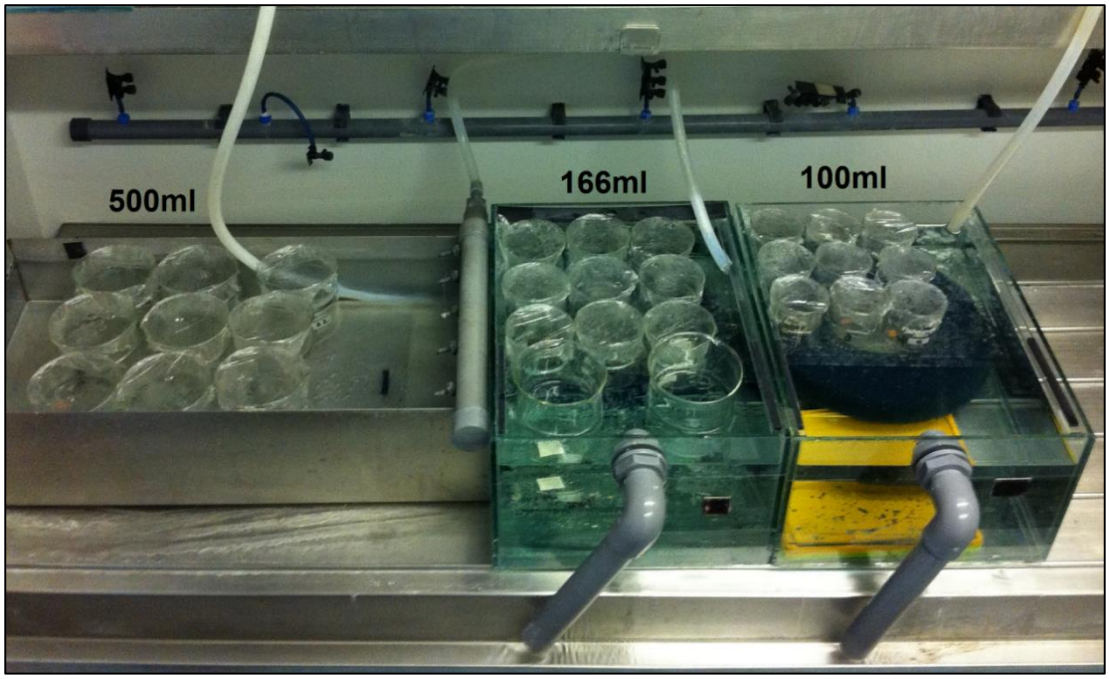


Figure 7.1. Photograph of pilot experiment setup with beakers grouped according to volume (but not water change frequency). *Temperature stability was maintained by standing the beakers in tanks of heated water*

Table 7.3. Examples of different variables from studies which have exposed *B. glabrata in vivo* under both static and static-renewal conditions. *Variables not reported marked with a dash.*

Study	Density (snails per 100ml)	Size (mm), age, or stage	Chemical	Exposure length	Species	Water change frequency
Allah *et al*., (1997)	2	12	Heavy metals	6 week	*B.g*	-
Bakry *et al*., (2012)	1	-	Azadirachta indica plant	24hr	*B.a*	-
Iqbal and Sinha, (2011)	1.3	-	BPA	10 days	*B.g*	None
Mohamed, (2011a)	0.5	8-10mm	Roundup	7 days	*B.a*	-
Oliveira-Filho *et al*., (2009)	3.3	3 month old	nonylphenol	8 weeks	*B.t*	twice a week
Russo *et al*., (2008)	-	25	fomesafen	21 days	*Lymnea*	every 3 days
Salice and Roesijadi, (2002)	-	egg	Cadmium	2 weeks	*B.g*	-
Salice and Roesijadi, (2002)	10	6 week old	-	2 weeks	-	-
Salice and Roesijadi, (2002)	2	-	-	2 weeks	-	-
Serrano *et al*., (2002)	4	7-8mm	hydrocortisone	4 days	*B.g*	every 24hrs
Toledo and Fried, (2010)	5	adult	-		*B.g*	-
Thomas and Benjamin, (1974)	4-0.25	-	none	3 week	*B.g*	every 3 days
Gust *et al*., (2013a)	0.2	40mm	pharmaceutical mixture	3 days	*Lymnea*	daily
Gust *et al*., (2013b)	0.6	35mm	effluent	7 days	*Lymnea*	daily

7.2.3. Primary Study

The major difference between this study and previous reports of effluent exposures in molluscs is that the effluent was used in the form of a concentrated extract (in ethanol) rather than the original raw water. Primary effluent was obtained from a large London sewage treatment works (I am unable to disclose precise details due to a confidentiality agreement). There were two main reasons I chose to use an extract.

Firstly, the extract process also removes possible confounding factors such as large quantities of bacteria or other microbes. While a reasonable degree of bacterial contamination would be expected in a natural system, the length of my exposure period, the time between water changes and the tropical temperatures required to maintain *B. glabrata*, meant that a typical effluent is likely to have quickly become acutely toxic. Indeed, there are no previously reported studies in which tropical molluscs have been exposed to effluent, possibly for this very reason. Secondly, use of an extract enabled me to concentrate the sample above its normal level. River water measurements from transmission areas show contaminant levels that are considerably higher than in the UK, even though the actual chemical composition, especially in urban areas of more developed transmission countries like Brazil, is likely to be fairly similar. It is not unusual for levels of the same chemical to be found 10 times higher, or more, in transmission countries such as Brazil (Sodré, *et al*., 2010; Moreira, *et al*., 2011).

7.2.3.1. Exposure of *B. glabrata* to an effluent extract

Based on the results gained from the pilot study, I was able to begin the main study knowing the amount of effluent that would be required. For the final study, snail density was 5 per 166ml with water changes every third day.

7.2.3.1.1. Effluent Collection and Stock Preparation

Final effluent was collected from the sewage treatment works as composites in ~33L volumes on three separate days. Effluent was stored in 2.5L glass brown Winchester bottles and kept in a cold room at 4^{o}C. The day following collection effluent bottles were taken individually from the cold room and the contents pre-filtered through 100µm silicon mesh into a clean glass bottle of equal volume (Figure 7.4 A) while kept on ice.

7.2.3.1.2. Solid phase extraction; disk conditioning and effluent extraction

47mm Octadecyl C18 extraction disks (Empore^{TM}) were secured into the manifold and carefully layered with 1cm of Filteraid glass beads (~50µm, Empore; Figure 7.2 A). To prime the new disk, 10ml of absolute methanol was added and allowed to run through the disk under vacuum pressure until dry (Figure 7.2 B-C). After ~5 minutes the vacuum pump was turned off, a further 10ml methanol was added, of which 1ml was allowed to gently run through the disk before closing the valve and letting the disk soak in the remaining 9ml for ~30 seconds (Figure 7.2 D-E). After soaking, the valve was again opened and all but the last 1ml methanol was allowed to run through before closing the valve (Figure 7.2 F). ddH_2O was added to the 1ml of remaining methanol to make a final volume of 10ml and the valve was quickly reopened allowing the methanol and all but 1ml of the diluted methanol to pass through (Figure 7.2 G-H). This stage of conditioning requires careful attention to avoid the disk drying out. The disk must remain immersed in liquid at all times while attempting to substitute the methanol for water. For the final stage of the filtration process, effluent was poured on top of the water layer to a final volume of 250ml (Figure 7.2 I) and the valve and vacuum pump were turned on to draw the effluent through the conditioned disk. This process was performed simultaneously for each of the six extraction ports (Figure 7.2 B). When effluent levels were running low they were replenished before the disk dried, until all of the effluent had passed through. Typically, the disks required changing after every 1-3L due to gradual blocking which causes a substantial decrease in filtering rate.

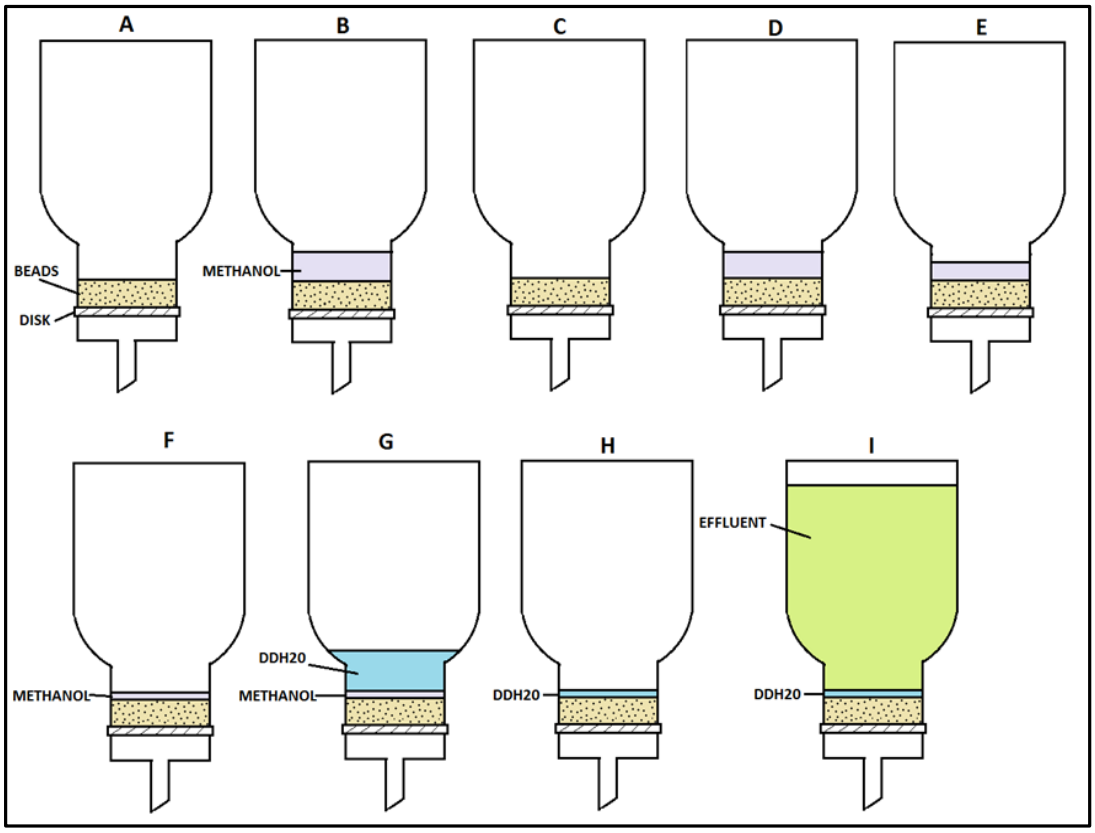


Figure 7.2. The process of priming and conditioning a C18 disk and extracting an effluent sample through it. *A) Glass funnel containing filteraid beads and C18 disk B) Addition of methanol to the funnel C) Methanol has been extracted through the disk D) More methanol added to the funnel E) 1ml of methanol has been allowed to pass through the disk F) All but ~1ml of methanol has been allowed to run through the disk G) Double-distilled water is added over the methanol H) Methanol and majority of the water are allowed to pass through the disk I) Effluent sample is added over the remaining water and extraction of sample is ready to begin.*

7.2.3.1.3. Solid phase extraction; disk elution and stock creation

Following extraction, the used disks were left to air dry under vacuum for ~30 minutes (Figure 7.3 A). The pump was turned off and the glass apparatus (as well as the disk) was removed from the manifold in one piece with the clamp in place. The outlet of the metal manifold was placed inside a glass collection tube (15ml) placed in such a way as to not obstruct the air flow. The glass apparatus was then reattached so that the outflow would run into the glass collection tube (Figure 7.3 B). With the valve closed, 10ml of methanol was added to the disk and 5ml allowed to run slowly through into the collection tube, followed shortly by the remaining volume of methanol to ensure maximum recovery of compounds from the disk (Figure 7.3 C-

E). At this point the glass collection tubes were sealed with screw-tops and Parafilm and stored at 4°C and the eluted disks discarded.

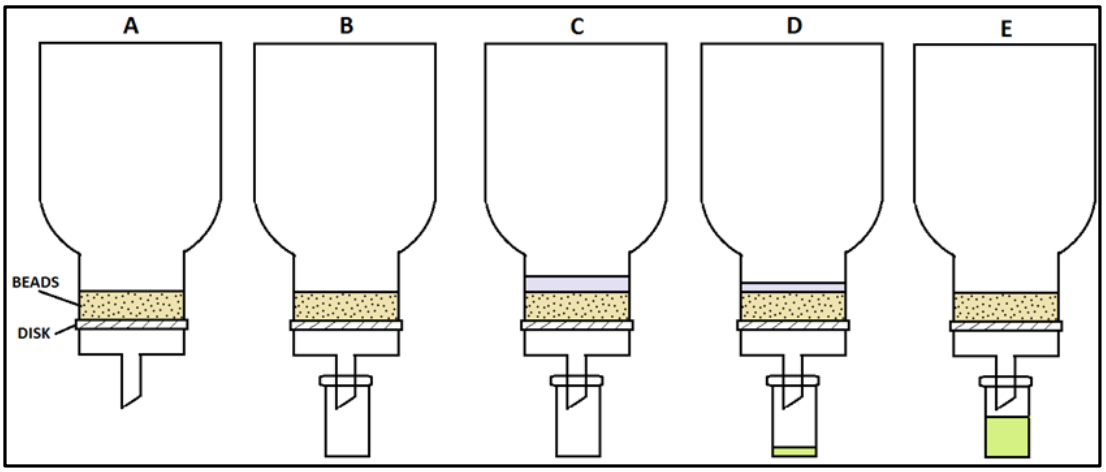


Figure 7.3. The process of eluting the bound effluent chemicals from the C18 SPE disk. *A) After the effluent sample has passed through the C18 disk is allowed to dry B) Glass tube placed beneath the funnel to collect the eluted sample C) Methanol is added to the disk D) Half of the methanol is allowed to pass through the disk and enter into the collection tube with the eluted chemicals E) The remaining methanol is allowed to pass through and all of the eluted effluent extract is now contained in the glass tube.*

Completion of the extraction process yielded a concentrated effluent extract in 100% methanol. The collection tubes were added to a TurboVap for drying at 50°C under nitrogen (~15 bar). Samples were periodically checked until they reached 'incipient dryness'; the point at which the methanol had essentially evaporated with only enough remaining to leave a viscous residue (rather than a completely dry sample) that could be more easily dissolved in ethanol (Figure 7.4 C). Finally, 1ml of ethanol was added to each centrifuge tube, the tubes were mixed vigorously until no obvious residue remained and the concentrated extracts then pooled together to form the final stock of known concentration relative to the original sample (Figure 7.4 D).

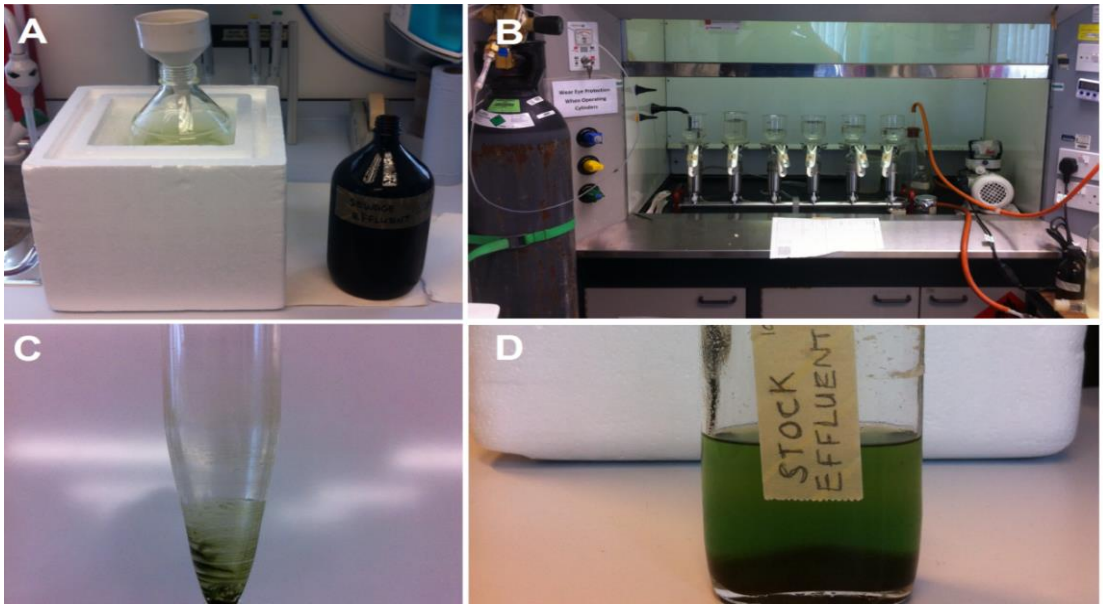


Figure 7.4. Photographs of the effluent extraction process. *A) Pre-filtration; the effluent is contained in a 2.5L brown glass bottle and will be poured through a pre-filter funnel which is placed on top of a clear bottle in an ice container B) The multi-port manifold is under a fume-hood and connected to a pump ready for extraction C) The methanol has been evaporated leaving behind a residue containing the effluent extract D) The final composite effluent stock in ethanol.*

7.2.3.1.4. Chemical Composition of Samples

Since we were unaffiliated with the STW from which we collected the effluent, it was not possible to disclose the known chemical composition of the samples based on the operators own monitoring data or undertake independent measurements ourselves. However, as effluent is a complex mixture, with a typical effluent containing in excess of 80,000 chemicals and their biotransformation products, the analytical chemistry data would only be of limited value in identifying the possible cause of any observed effects on immune suppression, infection and transmission.

7.2.4. Exposure Setup

To avoid the use of solvents within the exposure system, a method similar to that described by (Filby *et al.*, 2007) was adopted whereby an appropriate volume of effluent extract (in ethanol) was added directly to glass beakers and allowed to air dry before being re-suspended in 166ml fresh, filtered water of the kind our stock adult snails are kept in.

To ensure a complete developmental exposure, egg masses of approximately equal size were collected as soon after laying as possible and were subsequently ethanol rinsed. The eggs masses were placed in small glass tubes (10ml) and were exposed to the different effluent concentrations. To ensure sufficient numbers of snails for the experiment, I used two egg masses per tube. After hatching, snails from each dose were transferred with a paint brush to 6 replicate beakers (Figure 7.5). During the entire exposure in the beakers, the snails were fed and temperature maintained in the same way used for the pilot study and exposure water was changed every third day in the same manner.

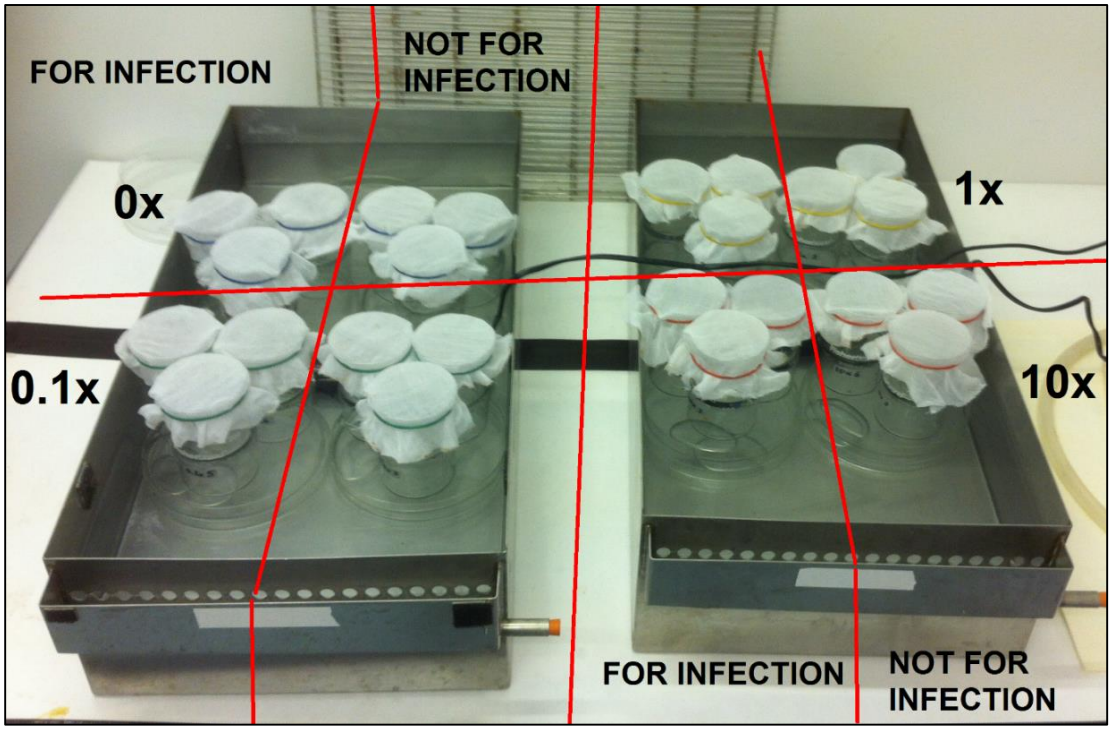


Figure 7.5. Photograph of the main study setup for adult snail exposure to the effluent extract**.** *The red lines indicate the grouping of the beakers within each tray; a row of 6 represents beakers all from the same effluent treatment and the 3 on the left of the vertical line are due for infection with S.mansoni while those to the right will remain uninfected.*

After the effluent exposure period was complete, half of the snails from each treatment were taken for infection at the London School of Hygiene and Tropical Medicine. To account for uneven mortality in the beakers, numbers were reduced to three snails per replicate 'group' and therefore 9 snails/treatment were designated for infection. For practicality and health and safety reasons I was unable to infect snails

at Brunel and was unable to continue the effluent exposure at LSHTM. As a result, I chose to end effluent exposure at this stage and infected and uninfected snails were maintained in freshwater for the final duration of the infection study. Since effluent volume was no longer a factor, snails were transferred to tubs and the water volume was increased (Figure 7.6). Snails remained in the same groups as from when they were exposed in the beakers and were separated within the tub by stainless steel gauze.

Conditions for infected and uninfected snails were kept as close to identical as possible, within the limitations of the experiment. Due to the stress of infection, oxygen lines were added to reduce mortality at LSHTM, and consequently the same was done to the non-infected group at Brunel. Both groups were kept in the dark as this is necessary to prevent pre-mature shedding in the infected group.

The infection process was as described in Chapter 2.

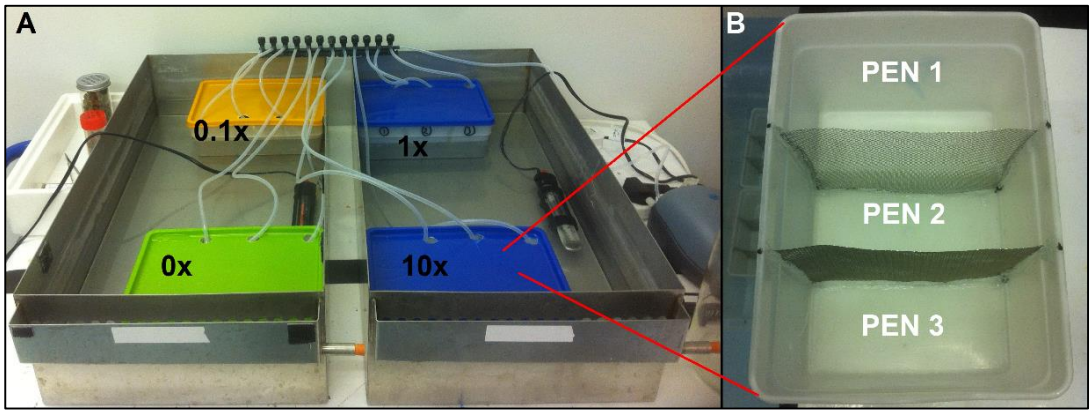


Figure 7.6. Experimental setup for infected (and uninfected) snails after the period of effluent exposure. *A) Each tub represents snails which were previously exposed to a particular effluent concentration B) Each pen represents an experimental unit.*

7.2.5. Experimental Endpoints

After the infections had been allowed to mature, the infected and uninfected snails were allocated to various endpoint categories. Of the remaining snails, one quarter (twelve) snails (one from each pen) were used for either shedding, histology, MTT assay or motility assay.

Details of the shedding process can be found in Chapter 2 (Section 2.3.6.4). The MTT and motility assays were performed essentially as described in their respective chapters, except for alterations in handling due to the infection status of one half. To maintain consistency between samples infected and uninfected, all snails (regardless of treatment and location) were treated in the same way. Infected snails could not be taken back to our laboratory and so hemolymph was quickly drawn at LSHTM from snails maintained in the dark and the filtering process previously described ensured that no cercariae could contaminate the hemolymph. Samples were kept on ice during transport to the Brunel laboratory for processing and all assays commenced approximately one hour after hemolymph extraction. For the MTT assay, zymosan was added to all samples (rather than half the samples as was the case during method development).

7.2.5.1. Histology

12 of the *S.mansoni*-exposed snails were used for histology. Snails were euthanized in magnesium chloride and then placed (still in their shells) into individual tubes containing pre-prepared Bouin's fixative solution (5% acetic acid, 9% formaldehyde, 9%, 0.9% picric acid in dH_2O; Sigma). This process took place before snails had the opportunity to shed cercarie (a process which would significantly disrupt their tissue). After 24 hours in Bouin's the snails were transferred to 70% IMS and sealed for transportation. Histology was performed by Dr. Burkard Watermann (LimnoMar, Hamburg, Germany).

Dehydration and embedding in paraffin was performed automatically (Hypercenter, Shandon). Sections of 2µm were prepared from the paraffin blocks and these sections were taken at a minimum of two levels or more, with a distance of 200µm. Histological evaluation was performed on hematoxylin-eosin-stained slides (Zeiss Ultraphot) and photographs were taken with a digital camera (Olympus C5050).

Of each snail, 12–15 sections were taken and parasitic stages were counted. Histologically, the basis for evaluation of parasite stages was determined according to the following criteria:

- **Mother sporocyst**: round or oval cell mass with numerous daughter sporocysts, composed of undifferentiated basophilic cells
- **Daughter sporocysts**: undifferentiated basophilic cells in mother sporocyst with differentiation into cercariae
- **Cercariae**: proliferating cell masses in daughter sporocysts, differentiating into tissues of cercariae, muscles, nervous tissue etc.

7.2.6. Statistical Analysis

Due to the number of variables under consideration and the absence of a clear hypothesis, the pilot study was initially analysed by principle components analysis for data reduction. The remaining variables from the PCA which proved to be correlated were further analysed by linear regression.

Shedding and internal parasite quantity were analysed by linear regression, where counts were treated as continuous due to the large size of the count scale and the absence of zero values.

MTT was analysed as previously described (Chapter 5, Section 5.2.7), but infection status replaces zymosan +/- as a binary predictor variable. Motility was analysed by multiple regression due to the inclusion of infection status as a variable as was snail mortality.

Linear regression was performed in Graphpad Prism 6.0 and PCA and multiple regression were performed in IBM SPSS 22.0.

7.3. Results

The following section describes results from both the density experiment (pilot study) and the main study (snail size, mortality, cercarial shedding, hemocyte motility, hemocyte viability *via* MTT and infected snail histology).

7.3.1. Pilot Study

Following principle components analysis, with all variables included, the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy gave a value of 0.494. According to Kaiser (1974) values below 0.5 are unacceptable. 'Water change frequency' was removed as a factor since it had no acceptable correlation with any of the other variables r = <0.3. After removing 'water change frequency' the KMO value increased to 0.592 and Bartlett's test of sphericity was significant (p = <0.001). After viewing the anti-image correlation, I ultimately found that only two variables remained with KMO >0.5, these were Volume (0.544) and Size (0.7). Linear regression was performed on the remaining variables and predictably a strong negative correlation coefficient was found between density and snail size (r^2 = 0.6358, Y = -722.8*X + 2414; Figure 7.7).

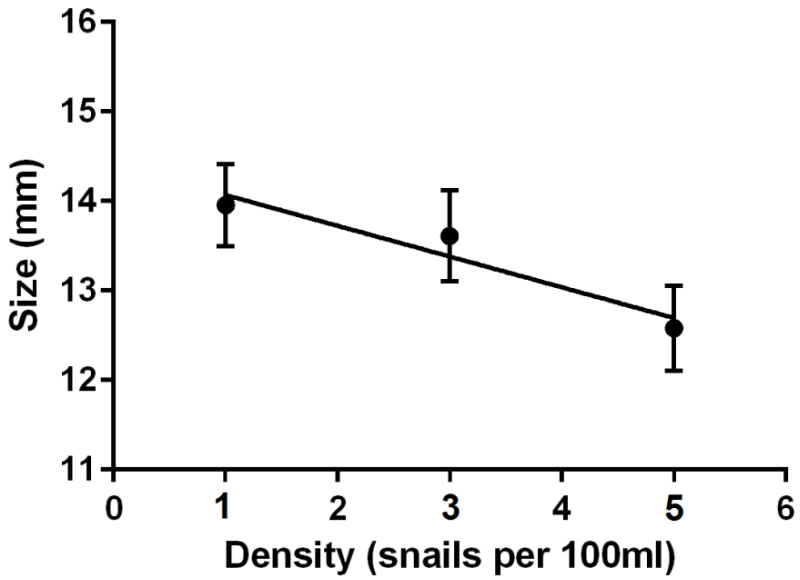


Figure 7.7. Linear regression model of the relationship between snail density and size. *Density is reported as the number of snails per 100ml water and size is reported as the length of the snail shell in mm.*

These results suggest that the studied water change frequencies did not significantly correlate with the other factors and that density was of most concern to the final study. In order to determine whether statistically significant results existed between the three different densities and their associated mean sizes Tukey's HSD was

performed. The multiple comparison test showed that both 166ml and 500ml volumes produced snails significantly larger than 100ml, but not from each other.

Table 7.4. Summary of mean size (mm), weight (g) and % mortality according to snail density (water volume in ml) and water change frequency. *n = 5 snails per beaker. Volumes adjusted according to desired density (100ml equivalent to 5 snails per 100ml; 166ml equivalent to 3 snails per 100ml; 500ml equivalent to 1 snail per 100ml).*

Beaker	Density (snails per 100ml)	Water change freq.	Mean Size (mm)	Mean Weight (g)	% Mortality (count)
1_3	5 per 100ml	daily (1)	12.042	0.386	13% (2)
4_6	5 per 100ml	every other (0.5)	12.935	0.438	7% (1)
7_9	5 per 100ml	third day (0.333)	12.771	0.427	20% (3)
10_12	3 per 100ml	daily (1)	13.052	0.481	27% (4)
13_15	3 per 100ml	every other (0.5)	14.051	0.568	33% (5)
16_18	3 per 100ml	third day (0.333)	13.722	0.486	27% (4)
19_21	1 per 100ml	daily (1)	14.123	0.577	20% (3)
22_24	1 per 100ml	every other (0.5)	13.438	0.507	7% (1)
25_27	1 per 100ml	third day (0.333)	14.306	0.570	27% (4)

7.3.2. Primary Study

Based on the results of the pilot study I chose to perform water changes on every third day, as performing changes more frequently did not appear to have any significant benefits regarding snail size or mortality rates. Density, however, was associated with size. Since there was a significant difference between 100ml volume and both 166ml and 500ml (but not between 166ml and 500ml) I selected a volume of 166ml (or density of 3 snails per 100ml) for the final study, especially as using 500ml would not have any significant impact on size, but would greatly increase the amount of effluent required to extract.

7.3.2.1. Size; post-exposure, pre-infection

After the exposure period of 4 weeks, and prior to infection of snails, measurements were taken for shell length. The results of a linear regression analysis between shell length (mm) and effluent concentration showed a strong negative relationship which

suggests that snail shell length decreases with increasing effluent concentration (r^2 = 0.6264, Y = -1.359*X + 7.262).

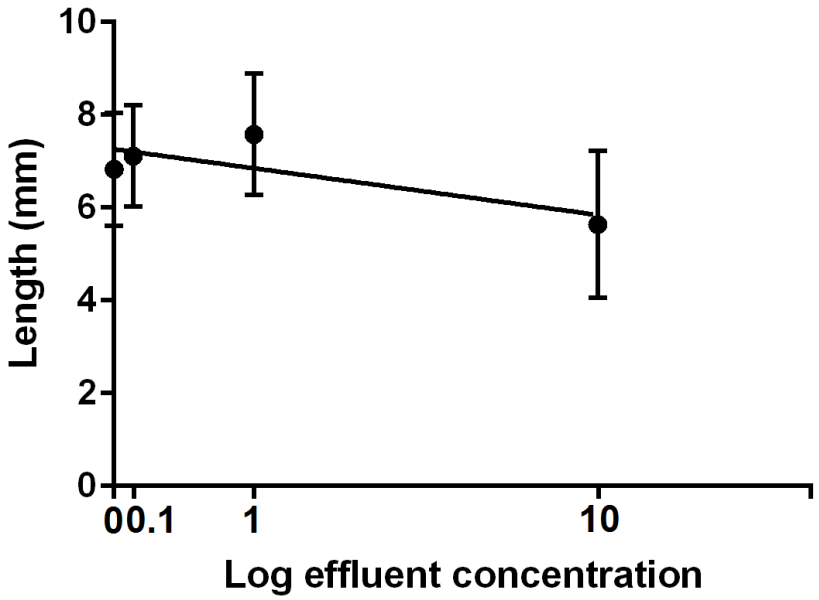


Figure 7.8. Linear regression analysis of the relationship between log effluent extract concentration and snail shell length (mm). *Data points are plotted as the mean of 6 independent replicates ± SEM.*

7.3.2.2. Mortality

Multiple regression was performed on mortality data, with infection status and dose as independent variables and percentage mortality as the dependent. Test assumptions were met, including a collinearity tolerance value well within the acceptable range (1.0). The model showed a strong and significant result (Adjusted R^2 = 0.774. p = >0.001), however 'infection status' explained the majority of the variance in mortality and the coefficient statistics showed that only 'infection status' was contributing significantly to the model. These results (Table 7.5) suggest that infection status, but not effluent dose, was positively related to increased mortality.

Table 7.5. Summary statistics from multiple regression analysis model built to predict mortality, based on the snails' *S.mansoni* infection status (positive or negative) and the concentration of effluent extract (0, 0.1, 1, 10x - log_{10}) to which it was exposed *in vivo*.

Variable	B	SE_B	β
Intercept	-11.876	4.999	
Dose	0.451	0.369	0.121
Infection status	27.550	3.094	0.883*

**p < .05; B = unstandardized regression coefficient; SE_B = standard error of the coefficient; β = standardized coefficient*

7.3.2.3. Shedding

All the assumptions of linear regression were met and the analysis established that mean number of cercariae shed had a strong positive relationship with increasing effluent concentration, the former explaining 68% of the variance ($r^2 = 0.6819$, Y = 4092*X + 4793).

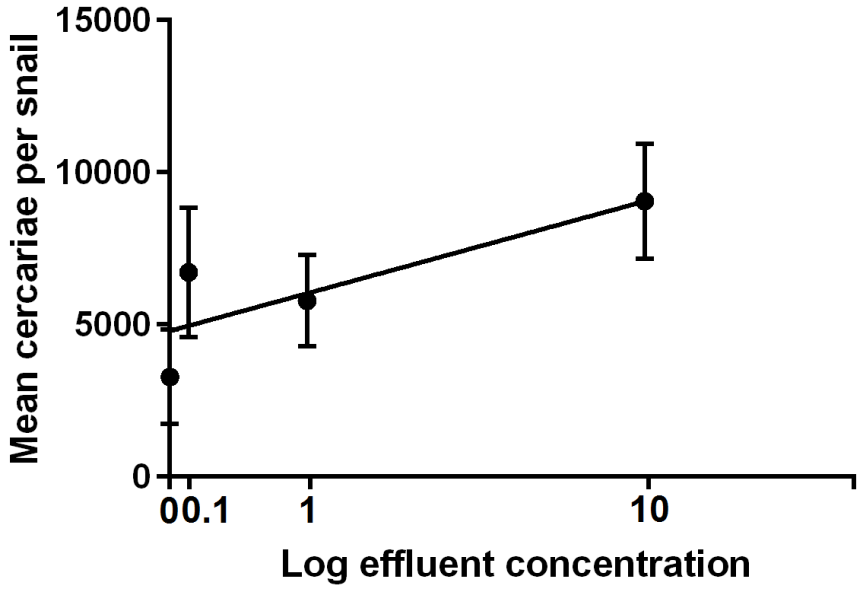


Figure 7.9. Linear regression model of the relationship between log effluent concentration and the mean number of cercariae shed per snail.

7.3.2.4. Motility

Multiple regression assumptions were met and the results showed a moderate statistically significant relationship between the independent variables of effluent dose and infection status and the dependent variable of cell velocity (µm/min) (Adjusted R^2 0.466; $p < .05$).

Table 7.6. Summary statistics from multiple regression analysis model built to predict motility (measured as mean velocity µm/min) of hemocyte cells taken from *B.glabrata*, based on the snails' *S.mansoni* infection status (positive or negative) and the concentration of effluent extract (0, 0.1, 1, 10x - $\log_{10}$) to which it was exposed *in vivo*.

Variable	B	SE_B	β	*p*
Intercept	3.308	0.338		0.000*
Infection status	-0.798	0.209	-0.581	0.001*
Dose	-0.068	0.025	-0.418	0.012*

*B = unstandardized regression coefficient; SE_B = standard error of the coefficient; β = standardized coefficient; p = significance of contribution to model; * = statistically significant (p-value < .05).*

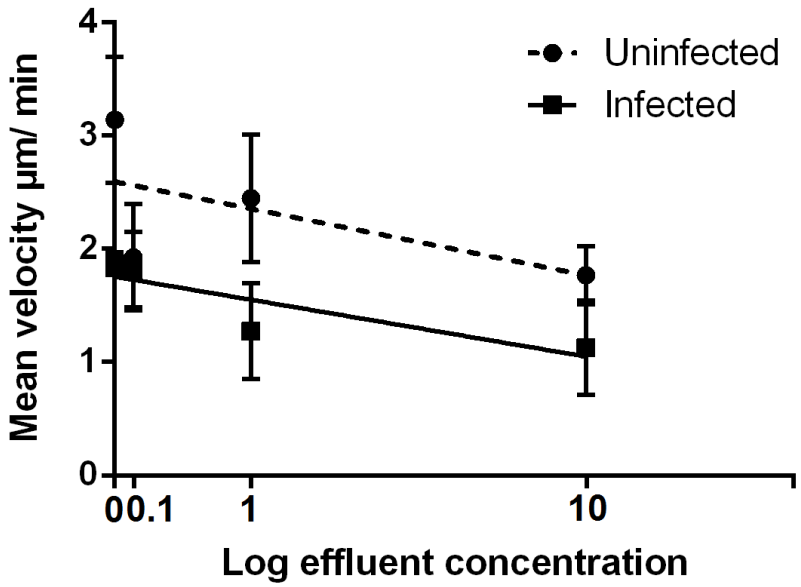


Figure 7.10. Visual representation of the multiple regression model built to predict *B.glabrata* hemocyte motility (mean velocity µm/min) based on the snails' *S.mansoni* infection status (positive or negative) and effluent concentration (0, 0.1, 1, 10x - $\log_{10}$). *Data points for mean hemocyte motility from uninfected snails are represented by circles and the regression line for these points is represented by a dashed line. Mean motility data points for hemocytes from infected snails are represented by squares and the corresponding regression line is solid.*

The results displayed in Table 7.6 suggest that *S.mansoni* infection of *B.glabrata* and exposure to increasing effluent concentration significantly reduce the motility of its hemocytes to a greater extent than either variable alone. Figure 7.10 shows that the regression lines for both infected and uninfected snails has the same gradient but with a different intercept, suggesting that the pattern of motility response to effluent dose was similar, but that the overall degree of mean motility was significantly less for hemocytes from infected snails i.e. increasing effluent dose reduces motility in snails, regardless of infection status, but infected snails have reduced motility to begin with.

7.3.2.5. MTT

Multiple regression was performed on the MTT assay data (Table 7.7) with infection status (binary) and dose (continuous) as predictor variables for absorbance reading (i.e. cellular/phagocytic activity). The relationship between the predictors/independent variables and the outcome/dependent was of moderate strength and indicated that positive infection status and increasing dose led to a reduction in cellular/phagocytic activity as displayed by MTT conversion and associated absorbance (Adjusted R^2 0.402; $p < .05$).

Table 7.7. Summary statistics from multiple regression analysis model built to predict phagocytosis (measured as mean absorbance) ability of hemocyte cells taken from *B.glabrata*, based on the snails' *S.mansoni* infection status (positive or negative) and the concentration of effluent extract (0, 0.1, 1, 10x - $\log_{10}$) to which it was exposed *in vivo*.

Variable	B	SE_B	β
Intercept	0.317	0.087	
Infection status	-0.128	0.054	-0.385*
Dose	-0.22	0.006	-0.553*

**p < .05; B = unstandardized regression coefficient; SE_B = standard error of the coefficient; β = standardized coefficient*

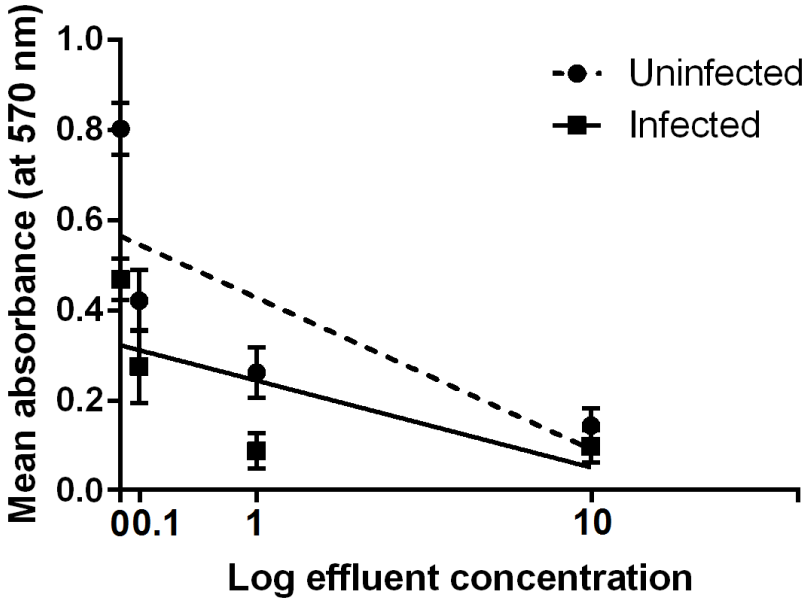


Figure 7.11. Visual representation of the multiple regression model built to predict *B.glabrata* hemocyte phagocytosis ability (measured as mean absorbance at 570nm) based on the snails' *S.mansoni* infection status (positive or negative) and effluent concentration (0, 0.1, 1, 10x - $\log_{10}$). *Data points for mean hemocyte phagocytosis from uninfected snails are represented by circles and the regression line for these points is represented by a dashed line. Mean phagocytosis data points for hemocytes from infected snails are represented by squares and the corresponding regression line is solid.*

7.3.2.6. Histology

Quantifying the number of mother sporocysts proved extremely difficult due to their similarity in appearance to daughter sporocysts and their scarcity in number (the number of mother sporocysts would be proportional to the maximum number of miracidia that infected the snail, so in my experiments this would be less than or equal to 7. As a result, we report daughter sporocysts (which would vastly outnumber mother sporocysts) and cercariae, which are easily distinguished from sporocysts and are also present in large numbers.

The number of parasites in infected snails was reported as being generally rather high across all doses. The numbers of miracidia in the mantle and tentacle tissue (their preferred penetration sites) were between 1- 5 suggesting that not all of the miracidia penetrated any snail, as is typical.

The numerical distribution of parasite stages within each snail is listed in Table 7.8.

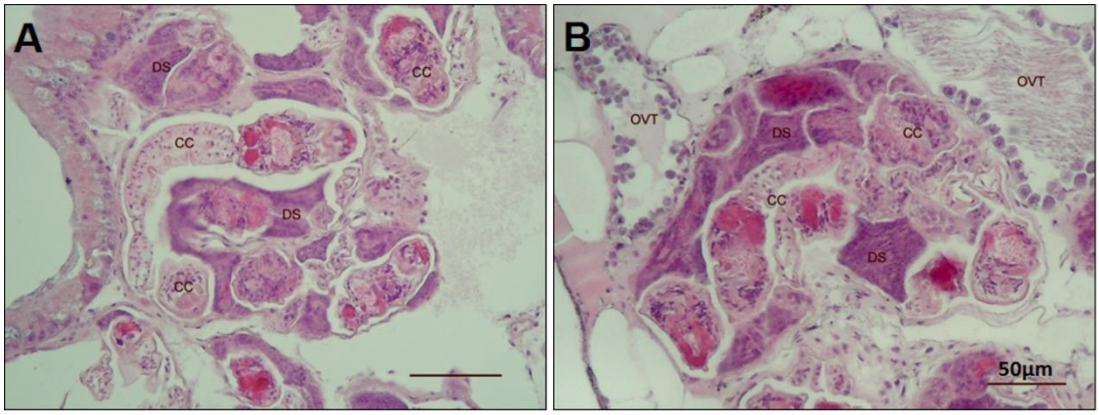


Figure 7.12. Histology images from snail 1, 0x; A) Digestive gland B) Gonad. Daughter sporocysts (DS), cercariae (CC), OVT = ovotestis, bar = 50µm.

Snail 1 contained numerous parasites at different stages within both the digestive gland (Figure 7.12 A) and the gonad (Figure 7.12 B). No host immune-reaction was apparent toward the parasites in either organ.

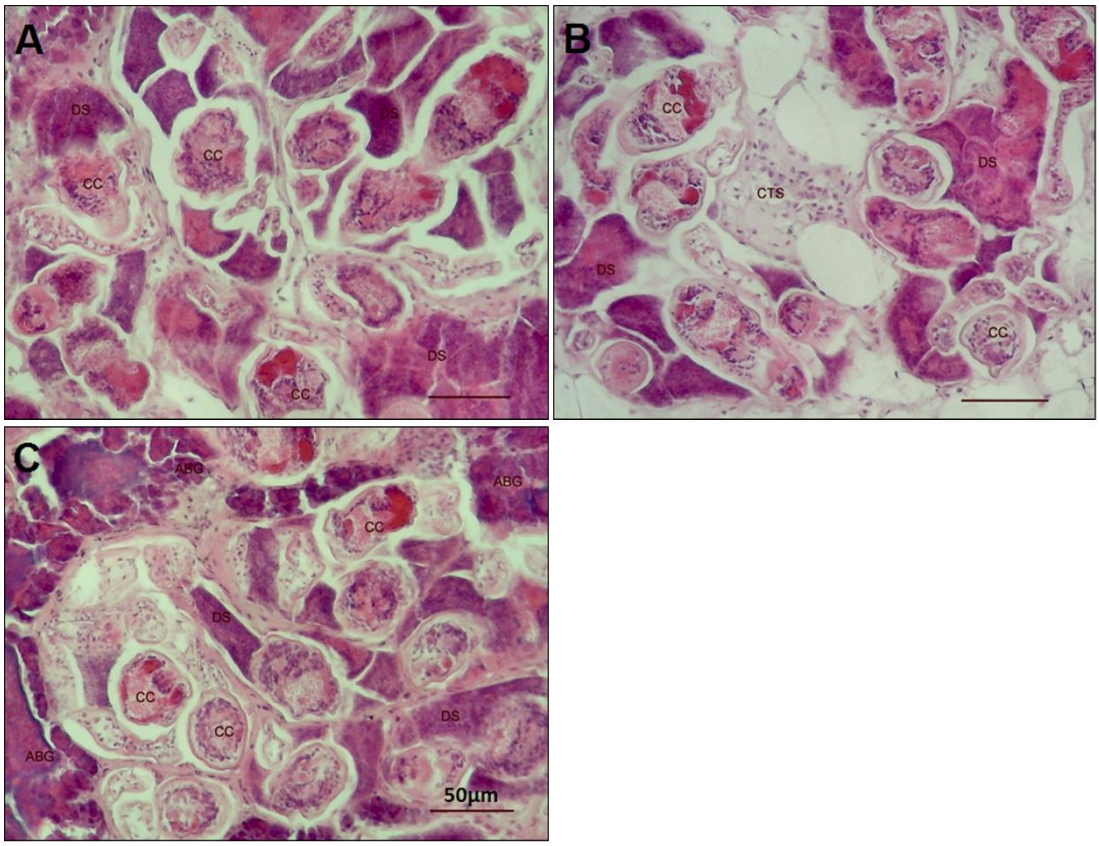


Figure 7.13. Histology images from snail 2, 0.1x; A) Digestive gland B) Gonad C) Albumen gland. Daughter sporocysts (DS), cercariae (CC) and ovotestis (OVT) connective tissue (CTS) albumen gland cells (ABG), bar = 50µm

Snail 2 contained numerous parasites with no observable host reaction in the digestive gland (Figure 7.13 A). There was evidence of slight infiltration of hemocytes in the connective tissue of the gonad (Figure 7.13 B). No host-response was apparent toward the daughter sporocysts and cercariae found in the albumen gland (Figure 7.13 C).

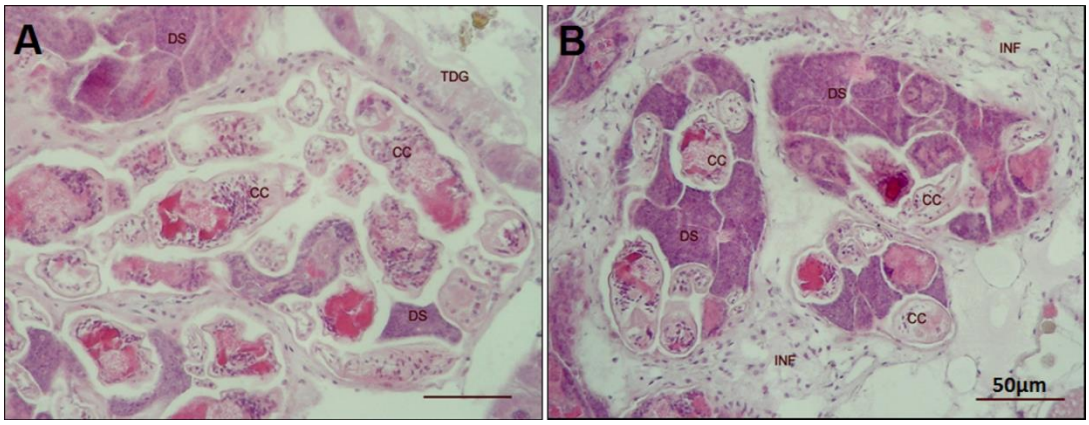


Figure 7.14. Histology images from snail 3, 1x; A) Digestive gland B) Gonad. Daughter sporocysts (DS), cercariae (CC), tubule of digestive gland (TDG), infiltration of hemocytes (INF), bar = 50µm.

Snail 3 contained numerous shrunken parasite stages in both the digestive gland and the gonad (Figure 7.14 A and B). The deformed parasites were likely related to a degree of host response shown by infiltrating hemocytes in the gonad (Figure 7.14 B).

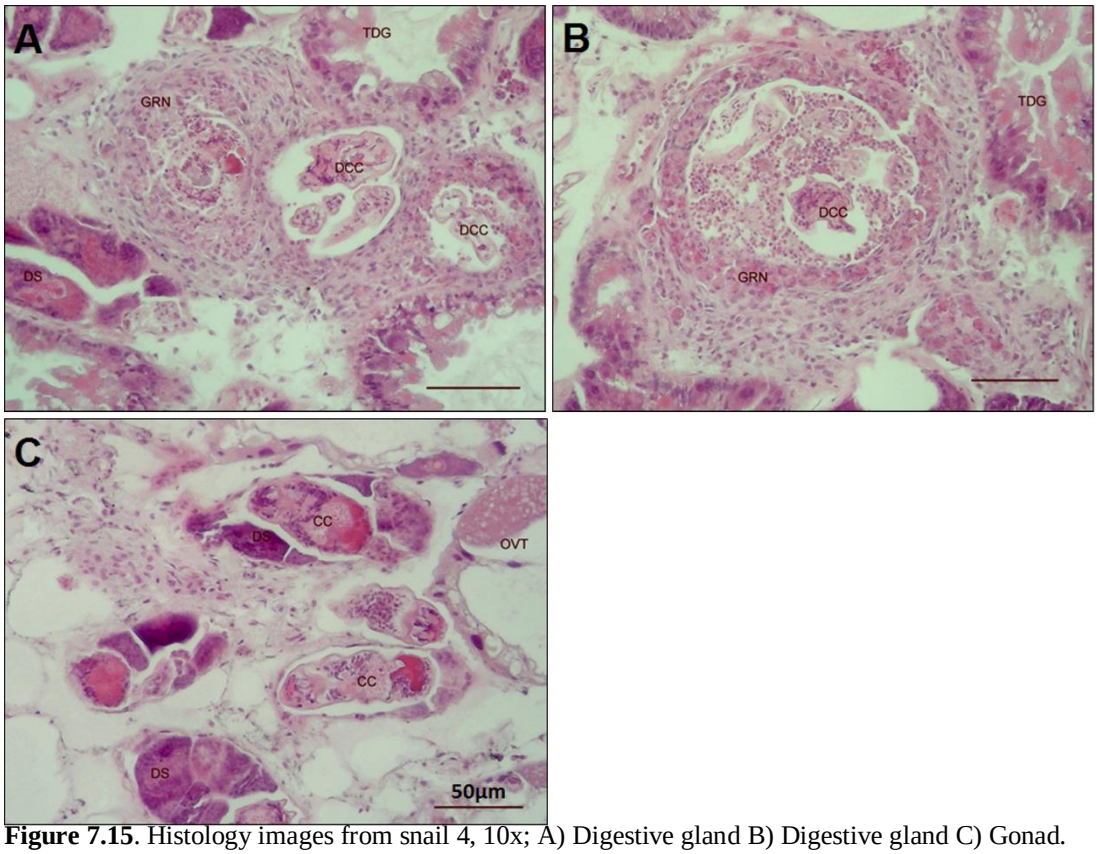


Figure 7.15. Histology images from snail 4, 10x; A) Digestive gland B) Digestive gland C) Gonad. Daughter sporocysts (DS), cercariae (CC), degenerated cercariae (DCC), granuloma (GRN), tubule of digestive gland (TDG), ovotestis (OVT), bar = 50µm.

Snail 4 exhibited multiple granulomas in the digestive gland (Figure 7.15 A and B) inside which were cercariae displaying degenerated features. Parasites were also present in the gonad tissue but with significantly less host reaction (Figure 7.15 C).

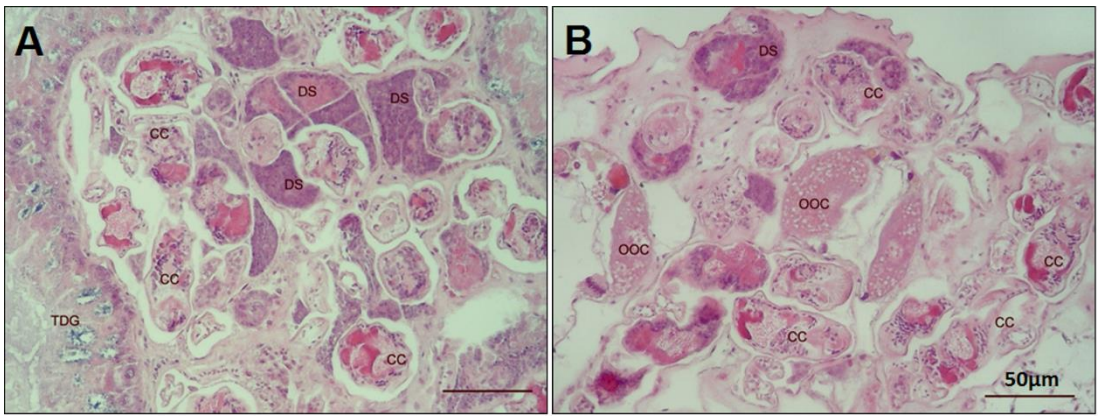


Figure 7.16. Histology images from snail 5, 0x; A) Digestive gland B) Gonad. Daughter sporocysts (DS), cercariae (CC), oocytes (OOC), tubule of digestive gland (TDG), bar = 50µm.

Snail 5 contained numerous parasites of healthy appearance in both the digestive gland and gonad and without evidence of host reaction (Figure 7.16; A and B).

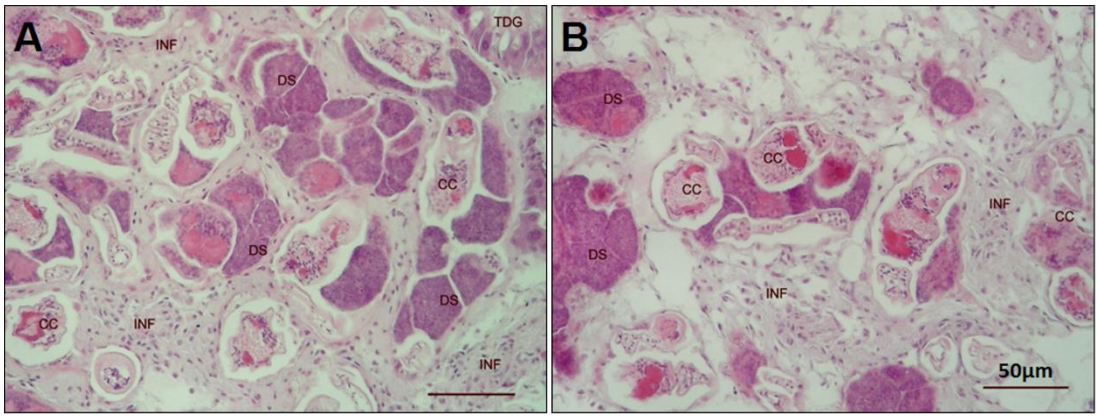


Figure 7.17. Histology images from snail 6, 0.1x; A) Digestive gland B) Gonad. Daughter sporocysts (DS), cercariae (CC), infiltration of hemocytes (INF), tubule of digestive gland (TDG), bar = 50µm.

Snail 6 displayed an immune reaction toward the parasites in both the digestive gland (Figure 7.17 A) and the gonad (Figure 7.17 B) as shown by infiltrating hemocytes in the interstitial tissue.

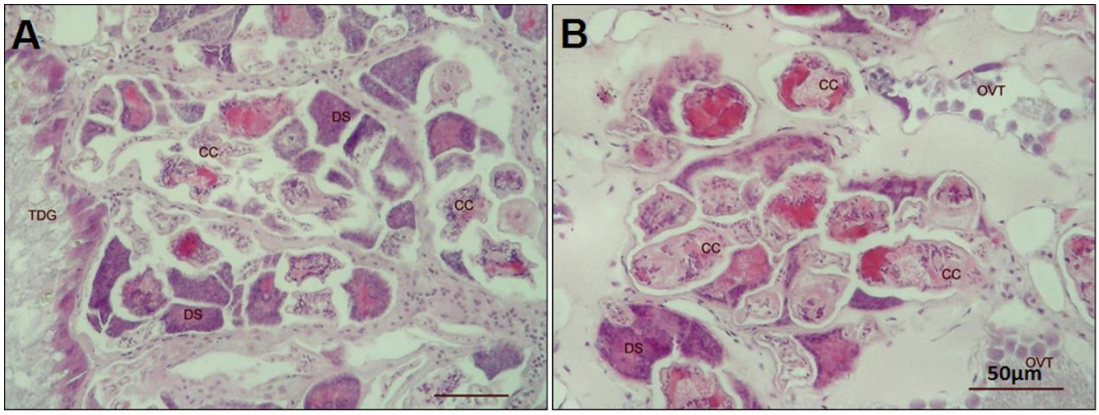


Figure 7.18. Histology images from snail 7, 1x; A) Digestive gland B) Gonad. Daughter sporocysts (DS), cercariae (CC), tubule of digestive gland (TDG), ovotestis (OVT), bar = 50µm.

No host reaction was apparent toward the parasites embedded in the digestive gland (Figure 7.18; A) or gonad (Figure 7.18; B) of snail 7.

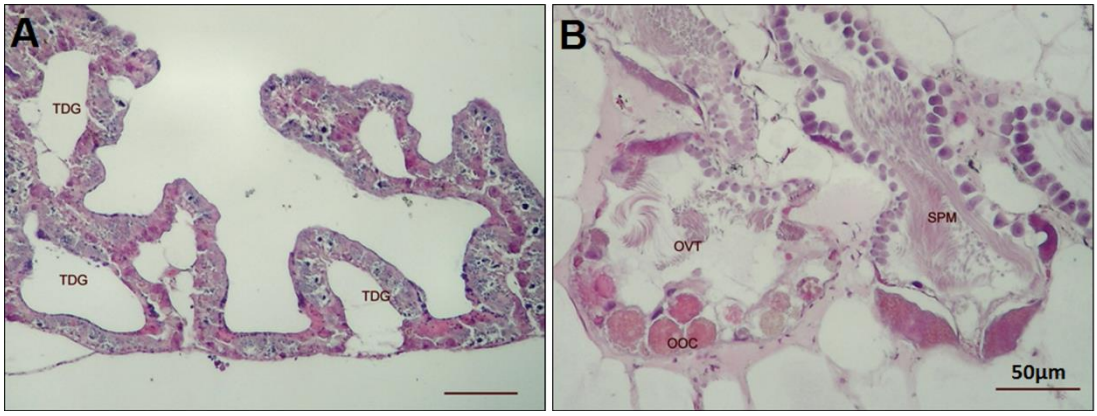


Figure 7.19. Histology images from snail 8, 10x; A) Digestive gland B) Gonad. Ovotestis (OVT), oocytes (OOC), tubule of digestive gland (TDG), mature sperm (SPM), bar = 50µm.

Snail 8 contained no parasites in the observed sections. The digestive gland of snail 8 was substantially distorted and the tubules were enlarged, extremely dilated and their epithelial cells flattened, all of which is indicative of toxic damage (Figure 17.19 A). When compared with the digestive gland of snail 5, a control snail, the extent of the difference in appearance is apparent (Figure 17.20 A and B). The difference in dilation of tubules is indicated by the comparative size of the lumen which is shown circled in Figure 17.20 (A and B) and accompanied by an anatomical diagram of the molluscan digestive tubule (Figure 17.20; C).

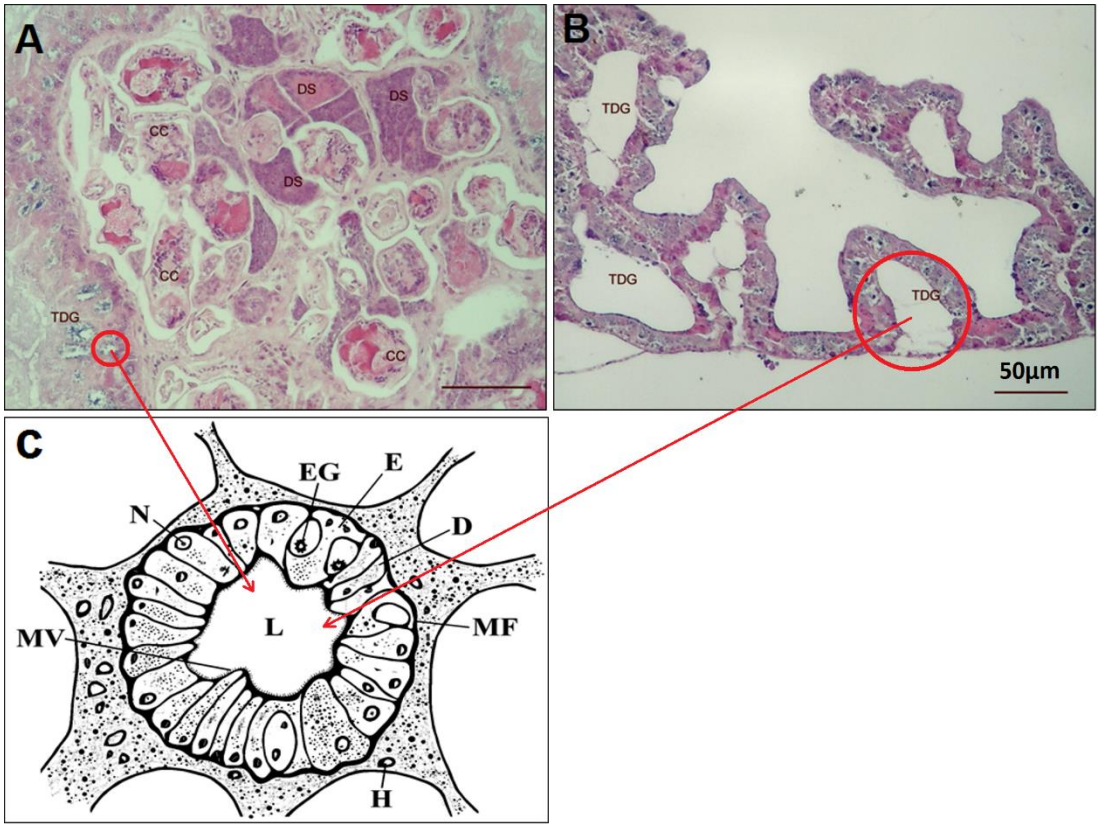


Figure 7.20. Comparison of digestive gland sections from snail 5 (0x) and snail 8 (10x) showing the extent of toxic damage. *A) Digestive gland of snail 5, cross-section of tubule circled in red B) Digestive gland of snail 8, cross-section of tubule circled in red C) Cross-sectional diagram of a molluscan digestive tubule, re-drawn and adapted from Hamed et al., (2007). Daughter sporocysts (DS), cercariae (CC), tubule of digestive gland (TDG), mature sperm (SPM), nucleus (N), excretory granule (EG), excretory cell (E), digestive cell (D), muscle fibers (MF), hemocyte (H), microvilli (MV), bar = 50µm.*

The gonad of snail 8 was active, with spermatogenesis and oogenesis apparent, but the organ was reduced in size (Figure 7.19 B).

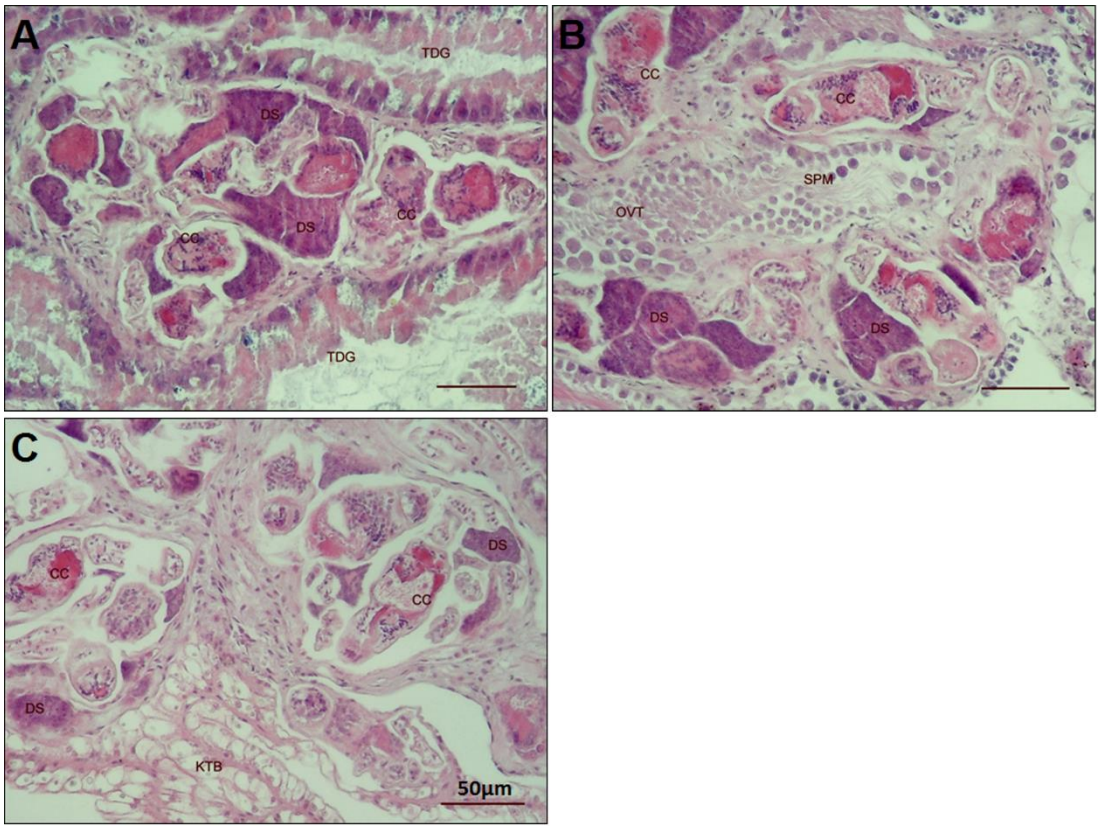


Figure 7.21. Histology images from snail 9, 0x; *A) Digestive gland B) Gonad C) Kidney. Daughter sporocysts (DS), cercariae (CC), Ovotestis (OVT), oocytes (OOC), tubule of digestive gland (TDG), mature sperm (SPM), kidney (KTB), bar = 50µm.*

Snail 9 showed large numbers of parasites in the digestive gland, gonad and kidney (Figure 7.21; A, B and C) but without any visible host reaction. The gonad displayed signs of proper function with spermatogenesis and oogenesis observable (Figure 7.21; B).

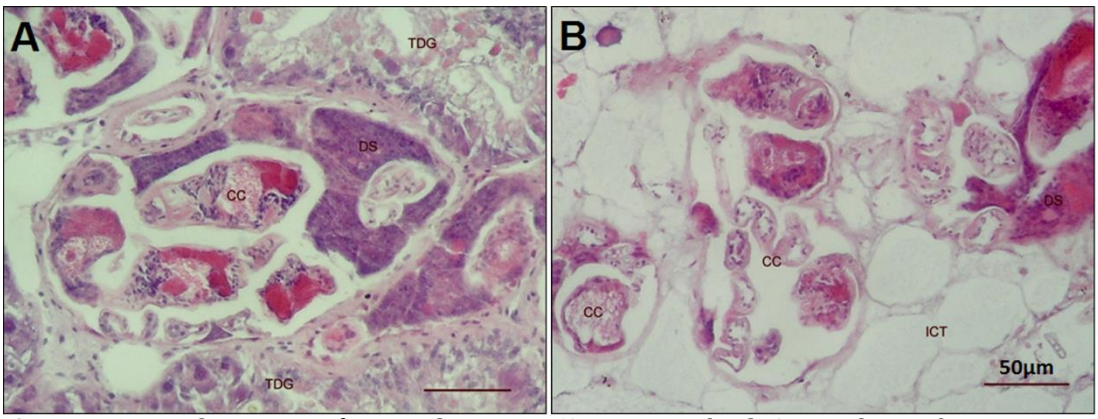


Figure 7.22. Histology images from snail 10, 0.1x; *A) Digestive gland B) Gonad. Daughter sporocysts (DS), cercariae (CC), tubule of digestive gland (TDG), interstitial tissue (ICT), bar = 50µm.*

In snail 10 cercariae were found in the digestive gland in relatively normal condition (Figure 7.22 A). In the gonad cercariae were quite small and displayed a shrunken body shape indicative of a host response (Figure 7.22 B).

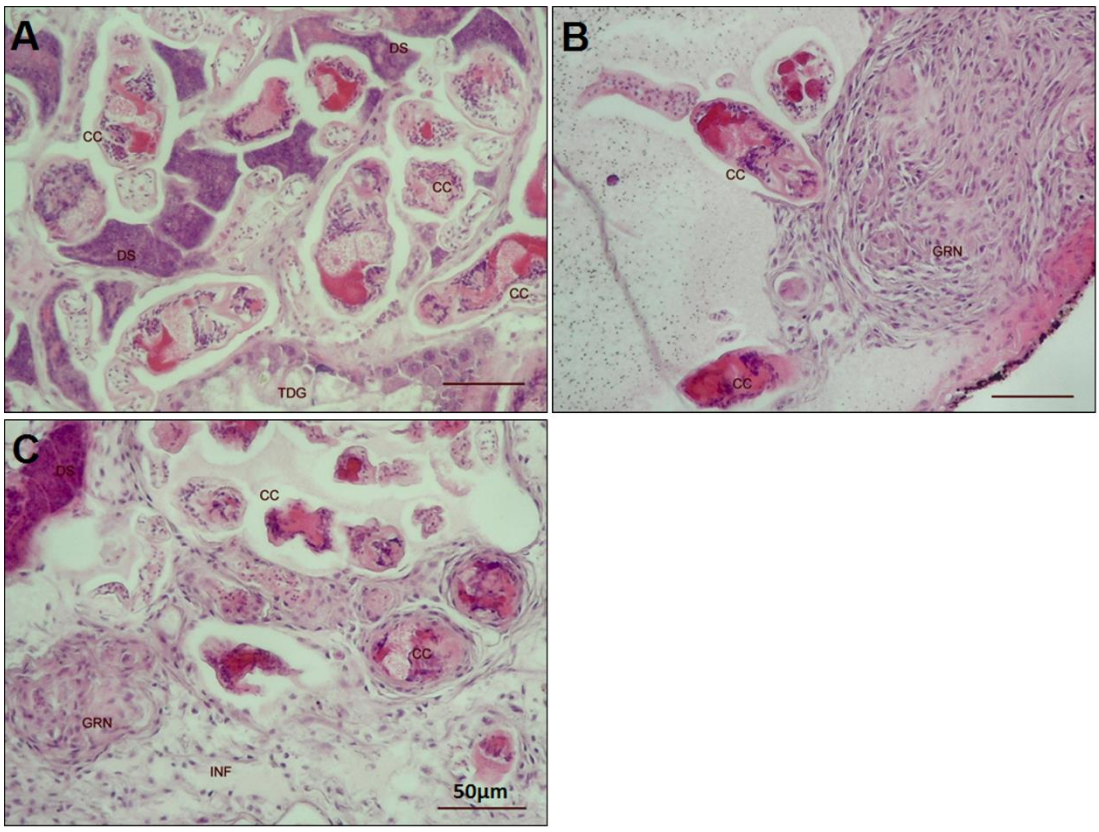


Figure 7.23. Histology images from snail 11, 1x; *A) Digestive gland B) Gonad C) Gonad. Daughter sporocysts (DS), cercariae (CC), infiltration of hemocytes (INF), tubule of digestive gland (TDG), granuloma formation (GRN), bar = 50µm.*

Snail 11 contained numerous parasites. The digestive gland and gonad contained shrunken cercariae (Figure 7.23 A, B and C). Multiple granulomas with capsule formation were present in the gonad, in association with high hemocyte infiltration, which is a result of an active host response (Figure 7.23 B and C).

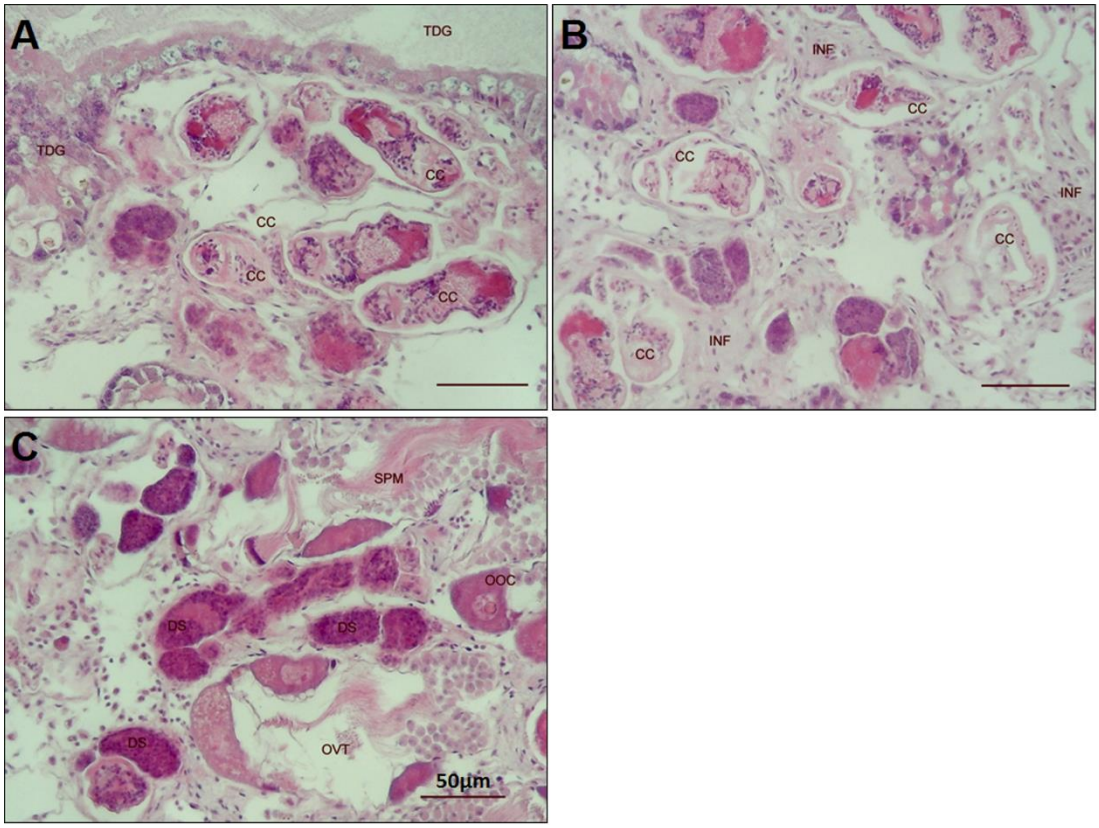


Figure 7.24. Histology images from snail 12, 10x; A) Digestive gland B) Gonad C) Gonad. Daughter sporocysts (DS), cercariae (CC), infiltration of hemocytes (INF), tubule of digestive gland (TDG), ovotestis (OVT), oocytes (OOC), mature sperm (SPM), bar = 50µm.

Snail 12 contained numerous cercariae inside the digestive gland without evidence of immune reaction (Figure 7.24 A). In parts of the gonad infiltration of hemocytes was present and associated with this immune-response were shrunken cercariae (Figure 7.24 B). In the other section of the gonad daughter sporocysts were present without apparent immune-response (Figure 7.24 C).

Table 7.8. Summary notes for each sectioned snail based on histological evaluation

Snail	dose	Figure	Histology notes
1	0x	7.12	Numerous parasites in digestive gland (A) and gonad (B) with no host reaction.
2	0.1x	7.13	Numerous parasites in digestive gland (A) and albumen gland (C) with no host reaction. Slight hemocyte infiltration in the gonad (B).
3	1x	7.14	Shrunken/damaged parasites of various stages in the digestive gland (A) and gonad (B). Evidence of hemocyte infiltration in the gonad (B).
4	10x	7.15	Multiple granulomas surrounding degenerated cercariae were present in the digestive gland (A and B). Considerably less reaction was present toward parasites in the gonad (C).
5	0x	7.16	Parasites present in the digestive gland (A) and gonad (B) with no host reaction.
6	0.1x	7.17	Infiltrating hemocytes and shrunken cercariae in both the digestive gland (A) and gonad (B).
7	1x	7.18	Parasites present in digestive gland (A) and gonad (B) with no apparent host reaction.
8	10x	7.19	No parasites were present in the observed sections. The digestive gland displayed signs of considerable toxic damage (A). The gonad was active, showing spermatogenesis and oogenesis, but reduced in size (B).
9	0x	7.21	Large numbers of parasites in the digestive gland (A), gonad (B) and kidney (C) but without any visible host reaction. Gonad was functional (C).
10	0.1x	7.22	Cercariae were found in the digestive gland (A) in a healthy condition but were found in a shrunken state, indicative of host response, inside the gonad (B).
11	1x	7.23	Shrunken cercariae were found in the digestive gland (A) and gonad (B and C). Multiple granulomas were present in the gonad in association with high hemocyte infiltration (B and C).
12	10x	7.24	Cercariae were present in the digestive gland without evidence of host-response (A). In parts of the gonad hemocyte infiltration and associated damaged parasites were found (B), while in other sections of the same organ parasites were present without host response (C).

It was not possible to include all the variables (dose vs number of daughter sporocysts and number of cercariae) in a single model without substantially violating assumptions of traditional statistical approches. Instead the total number of counted internal parasites was treated as continuous and used in a linear regression (as were the different stages separately). According to the replicate runs tests none of the

regression models showed relationships which differed significantly from a straight line (p = > 0.5). Effluent dose was able to explain only 0.05% of the variation in the total number of internal parasites in a snail (r^2 = 0.05074; Table 7.9). A similarly weak relationship was found between effluent concentration and the number of cercariae (r^2 = 0.006380; Y = -116.9*X + 856.5) whereas the number of daughter sporocyst showed a stronger, but still relatively weak, negative relationship to increasing dose (r^2 = 0.1087; Y = -605.9*X + 1558).

Table 7.9. Number of daughter sporocysts, cercariae and a combined total number of parasites for each snail/dose.

Snail	Dose	Daughter	Cercariae	Total
1	0	1339	243	1582
5	0	3186	2507	5693
9	0	1002	600	1602
2	0.1	1613	817	2430
6	0.1	694	348	1042
10	0.1	1679	707	2386
3	1	1649	926	2575
7	1	1340	684	2024
11	1	765	678	1443
4	10	2046	1361	3407
8	10	0	0	0
12	10	867	921	1788

7.4. Discussion

Results from the pilot study suggested snail density to be the most important factor in subsequent snail size (shell length in mm). Density-dependent growth is a well know phenomena in many species and is known to be the case in *B.glabrata.* The aim of the pilot study was to provide information on the minimum requirements for the snails in-terms of water change frequency and volume; the two factors that affect the final volume of effluent required for extraction (a significant limiting factor). These two variables were prioritised not only due to their relation to effluent volume, but also because (i) density is a known factor in snail size, which in turn is a factor in

survival and infectivity (endpoints in the main study) and (ii) water change frequency is related to other potentially important factors such as waste accumulation, oxygen level and pH. Based on the results from the pilot study a volume of 166ml (3 snails per 100ml) was chosen for the final study. While density showed a positive linear relationship with size (Figure 7.7), there was no significant difference between the top two densities (166ml and 500ml), so the lower of the two was chosen in order to reduce the amount of effluent needed. The density range only extended to 500ml (1 snail per 100ml) and it is possible that size would continue to increase with higher volumes/lower densities. However, lowering the density further would have required unfeasibly large volumes of effluent to extract. Thomas and Benjamin, (1974) suggested 1 snail per 100 ml (10 per litre) as the maximum density to support optimal growth, given that density related positive feedback does occur so large volumes are not necessarily always better. Their suggestion for optimal density is the same as the maximum in my pilot (10 per litre/5 per 500ml) and since this volume did not differ significantly from 166ml, it was decided that this density (3 snails/100ml) would be appropriate. Volumes for the final study were increased immediately after the effluent exposure during the infection phase since infected snails are known to benefit from lower densities (Tucker *et al.*, 2013).

Water change frequency was not found to be a significant factor in determining snail growth, at least within the range selected here. This is also not surprising since the accumulation of a certain amount of excretory material is believed to be beneficial, coupled with the fact that *B.glabrata* is well adapted to anoxic and DOM-rich water, being a pulmonate snail (Thomas and Benjamin, 1974; Moeller *et al.*, 2011).

In the main study, eggs were exposed within hours of being laid to ensure early developmental exposure because the early life stages of many species, including *B. glabrata*, are several orders of magnitude more sensitive to xenobiotics than adult stages (Oehlmann & Schulte-Oehlmann, 2002; Tallarico *et al.*, 2014). Developmental exposure therefore ensures greater potential for xenobiotic-induced effects and it is also more representative of field conditions where eggs would develop and hatch and the juvenile snails develop towards maturity in the same water conditions as adults.

Developmental exposure to certain xenobiotics has previously been reported to cause retardation of growth in other freshwater pulmonates (Khangarot and Das, 2010). This correlates with my findings of a general reduction in size based on measurements of shell length (a standard assessment of 'size') taken on all the study snails immediately post-effluent exposure. The impact of a reduction on size could have direct implications to transmission since Niemann and Lewis (1990) found that the percentage of snails which developed patent infections was higher in snails with smaller shell sizes.

Cercariae are usually located in the digestive tract which also appears to be the most prominent site of studied xenobiotic accumulation in molluscs (Couch and Fournie, 1993; Domouhtsidou and Dimitriadis, 2001; Oehlmann and Schulte-Oehlmann, 2002).

Damage to the digestive gland is typically indicative of chronic, rather than acute, exposure to environmental stressors due to its prominent involvement in bioaccumulation and excretion (Costa *et al*., 2013). More specifically, within the digestive gland the digestive tubules have been highlighted in several studies as sites displaying the most profound damage (Thompson *et al.,* 1974; Moore *et al.,* 1978; Couch 1984; Axiak *et al.,* 1988, Recio *et al.,* 1988), including molluscs exposed to effluents (Domouhtsidou and Dimitriadis, 2001), and have therefore been proposed as suitable biomarkers (Costa *et al*., 2013). The tubules have a similar function and appearance to the vertebrate intestine in that they are luminal and formed by a single layer of ciliated epithelial cells (Le Pennec and Le Pennec, 2001; Costa *et al*., 2013; Figure 17.20, C).

In my study, significant damage to the digestive tubules was found in the snails exposed to the highest (10x) effluent concentration. This type of damage was indicative of toxicity and not parasitism (Dr. Burkard Watermann, LimnoMar; personal communication), indeed the snail showing the greatest degree of toxic damage (snail 8, Figure 17.19 and Figure 17.20) was exposed but apparently never infected. The underlying mechanistic cause of the observed toxic damage to the digestive gland is difficult to determine due to the number of potential factors and their interplay, as well as the fact that the exact composition of the effluent was unknown. The fact that *B. glabrata* has been shown to be capable of absorbing

chemical mutagens from the aquatic environment suggests the interesting possibility that the digestive gland was improperly formed during growth and development and not simply damaged due to accumulation, although we cannot say what chemicals were present in my effluent extract, it is likely that there were mutagens due to their widespread use and pervasive nature (Canesi *et al*., 2007a; Tallarico *et al*., 2014)

In both infected and uninfected snails, multiple regression analysis of the MTT data revealed a moderate decrease in phagocytic activity with increasing dose, indicating that phagocytosis was being reduced as a results of effluent exposure *in vivo* (Table 7.7 and Figure 7.11). This is an interesting and informative result for two reasons. Firstly, the dose-dependent reduction in phagocytosis suggests an immunosuppressant effect of the effluent extract on the snails, something which pertains strongly to, but in itself doesn't fully answer, the central question of the thesis (i.e. it provides evidence of pollutant induced suppression of a component of the snail immune system). Secondly, since the same method was not able to detect any significant response to chemicals, including the effluent extract, exposed to hemocytes *in vitro* (Chapter 5; Section 5.3.1.3), we are perhaps offered evidence as to the importance of bioaccumulation. However, it is possible to suggest an alternative explanation in place of the second point. This result could instead be indicating a lower sensitivity to *in vitro* phagocytosis detection by the MTT assay compared with the Imagestream, since the latter did display a relationship between effluent exposure and phagocytosis *in vitro*, however this relationship was extremely weak (Adjusted $R2 = 0.06851$; (Chapter 5; Section 5.3.1.3) and the two assays were also analysed differently due to their respective study design.

Also apparent, but perhaps less surprising, was the significant overall reduction in phagocytosis rates among hemocytes from infected snails when compared to those from snails which were uninfected (Figure 7.11). Infection with *S.mansoni* has long been known to directly and specifically impair elements of the immune function of *B.glabrata,* including phagocytosis (Abdul-Salam and Michelson, 1980; Connors and Yoshino, 1990).

A final point of interest shown by Figure 7.7 is the convergence in phagocytosis rates between infected and uninfected snails at the highest effluent dose. This would perhaps suggest that phagocytosis is reaching its lowest possible functional level for

survival, but also again raises the possibility of indication of the lower level of detection for the assay.

Reduced phagocytic activity on its own does not necessarily directly cause damage to digestive gland cells, but it has been shown to be indicative of damage, particularly to digestive cell lysosomal membranes, since the process of phagocytosis is dependent on functioning cell membranes and lysosomes play a central role in the degradation of phagocytosed materials (Coles *et al.,* 1995 and Grundy *et al.*, 1996). In a study which exposed mussels to an artificial mixture of EDCs, designed to be representative of environmental levels, even a 0.1x concentration was able to induce a 55% decrease in lysosomal membrane stability, albeit in hemocytes rather than digestive gland cells (Canesi *et al.*, 2007b).

Therefore, in terms of a mechanistic cause, it seems feasible that the significant toxic damage seen in the digestive gland of snail 8 (Figure 17.19) was possibly as a result of irregular gene expression during developmental exposure or accumulation of xenobiotics and direct toxicity to digestive gland cells or possibly even both these factors together.

While the average number of parasites within snails remained relatively constant along the effluent exposure range, it was interesting to note that snails in higher effluent concentrations shed more parasites; a finding that may clearly have implications with respect to transmission of disease to humans. Increased shedding of cercariae from snails exposed to xenobiotics has previously been shown (Soliman, 2009; Kelly *et al.*, 2010), as has the opposite (Allah *et al.*, 1997; Morley *et al.*, 2003), however it is important to note that these studies investigated single chemicals from different classes (herbicides and heavy metals). Therefore, I can only state that chemicals typically contained in treated domestic effluent, such as that extracted for use in this study have, at environmentally relevant doses, previously been shown to affect cercarial shedding.

The mechanisms for xenobiotically-induced alterations in shedding are poorly understood, as are the mechanisms controlling cercarial emergence from snail tissue. The few studies which have almost exclusively only considered herbicides.

Hock and Poulin, (2012) found that *Potamopyrgus* snails infected with three different species of trematode shed between 1.5 and 3 times as many cercariae per

day when exposed to the herbicide glyphosate at levels considered to be sub-toxic to most aquatic organisms. Since a similar pattern of increased shedding was seen for all three species of trematode, and has also been observed previously in the same snail species exposed to the same chemical (Kelly *et al*., 2010) as well as different species exposed to the herbicide atrazine (Rohr *et al*., 2008), the authors suggest a 'general' weakening of the snail, and subsequent indirect benefit to the parasite, by herbicides as the cause, rather than something specific to a particular trematode or snail species (Hock and Poulin, 2012).

An important difference between my study and others is the fact that snails were infected after developmental exposure and were not exposed during infection. This was due to limitations in obtaining permission to use potentially human-infective snails in our laboratory or to take effluent to LSHTM. This fact warrants the consideration of other potential explanations for my findings.

Effectively this suggests a situation where none effluent-exposed parasites are presented to previously effluent-exposed, and potentially compromised, hosts. Since the snails were developmentally exposed (but not the parasites) we may hypothesise that the effluent-induced damage somehow enhanced the ability of cercariae to emerge without any direct and deleterious effects of effluent on the parasite associated with their accumulating xenobiotics. However, since the parasites are known to acquire resources from locations where xenobiotics are most heavily bioaccumulated, it is still quite possible that the parasites were indirectly exposed, although no supporting evidence could be found for this.

Equally, it may not matter whether the parasites are exposed or not, as in the studies performed by Rohr *et al*., (2008), Kelly *et al*., (2010) and Hock and Poulin, (2012), both parasite and host were exposed simultaneously yet the situation was apparently still more beneficial to the parasites as evidenced by their increased shedding rates. It is important to note that an increase in cercarial shedding is not necessarily indicative of increased transmission potential in the field, as others have shown reduced infectivity of cercariae from xenobiotic-exposed snails (Morley *et al*., 2003).

Both infection and increasing effluent concentration significantly contributed to a reduction in cell velocity/motility. *S.mansoni* is known to release excretory-secretory products which reduce *B.glabrata* hemocyte motility, although to my knowledge

these studies have previously only taken place on hemocytes exposed to ESPs *in vitro* (Lodes and Yoshino, 1990; Zelck *et al*., 2007). Reduced motility in hemocytes of mussels collected from contaminated sites has also recently been reported for the first time (Rioult *et al*., 2014). Here I have shown that the combination of infection and exposure to effluent decreases motility more than either factor alone. The ability to phagocytose and encapsulate are clearly dependent on motility. Zelck *et al*., (2007) showed that the ERK (extracellular signal-regulated kinase) signal transduction pathway in particular was crucial to all of these processes. The infection-induced reduction in phagocytosis which has been observed previously is associated with reduction in ERK phosphorylation (Zelck *et al*., 2007). Interestingly Canesi *et al*., (2006) and Porte *et al*., (2006) have both shown that ERK phosphorylation can be reduced by exposure to certain xenobiotics. Taken together these results suggest a synergistic or additive effect of infection and effluent exposure toward a reduction in cell motility (Table 7.6 and Figure 7.10) and subsequently phagocytosis (Table 7.7 and Figure 7.11), possibly induced by reduced ERK phosphorylation.

Infection was positively related with increased mortality, a phenomenon which is well documented in infected snails due to the associated physical trauma and reduction in nutrients (Eveland and Haseeb, 2011). Dose, however was not significantly associated with mortality.

Given my other conclusions this may suggest a scenario where physically/immunologically impaired snails are able to remain alive, possibly long enough to transmit more cercariae into their environment than if the combined burden of infection and exposure to effluent was more acutely fatal.

Chapter 8: *General Discussion*

The purpose of this research was to explore the broad question 'does pollution affect parasite-host interactions?' by considering the relationship between the freshwater snail *Biomphalaria glabrata* and its obligate parasite *Schistosoma mansoni.* Given the medical importance of this parasite-host relationship, and its tendency to occur in relatively polluted environments, it is surprising that of the numerous studies on immunotoxicology in aquatic molluscs, the vast majority have focused on commercial species only. Therefore, this project represents one of very few to explore the *B.glabrata – S.manosni* relationship in the context of ecotoxicology. Of the few studies that have investigated this particular hypothesis most, if not all, have focussed on single chemicals or chemical classes (heavy metals in particular) and single endpoints.

Given the rapid increase in urbanization of transmission countries like Brazil, their sewage effluents are becoming increasingly similar to those of western countries. However, while the effluent compositions are becoming more similar (pharmaceuticals, personal care products), the treatment levels still severely lag behind, often resulting in concentrations of as much as 10 fold higher than those reported in the UK. For these reasons, I decided to investigate the activity of a number of known immune/endocrine-modulators on key components of the *B.glabrata-S.mansoni* interaction.

There is a spectrum of investigatory models ranging from *in vitro* studies on cell lines to field studies and epidemiology (Krysko and Vandenabeele, 2009). Each approach has advantages and disadvantages, and in general, as the precision of measurement and control of variables reduces the environmental relevance increases and *visa versa*. Within the context of a laboratory-based project, a degree of environmental relevance was maintained through the use of primary cells and the inclusion of a static-renewal *in vivo* exposure.

In general, regression analysis was used to describe dose-response relationships rather than forcing the data into an ANOVA-type model which is a common procedure (Chapter 3, Section 3.3.7). While the latter approach can yield useful information, and has been used in this study in places, it violates the assumptions made by this kind of experimental design and appears to be mainly used to search for 'P-values' with respect to a black and white significant/not significant result.

Therefore, ANOVA-type analysis should be reserved for additional investigation and regression models adopted as the preferred first option in dose-response assays (Cottingham *et al*., 2005; Royston *et al*., 2006).

Given the difficult nature of primary molluscan hemocytes, and a relatively small body of work to build on, it was necessary to develop novel investigative approaches as well as make adaptations to existence techniques to address the research question. As a result, a significant proportion of this thesis involves method development and validation, rather than purely data generation.

For the study of the encapsulation interaction, a simple and inexpensive version of an established assay was adapted to accommodate xenobiotic exposure; the first attempt to do such an assay to my knowledge. Observations of the interactions obtained in my experiments compared favourably to those from previous *in vitro* assays with the same cell type (Castilllo and Yoshino, 2002; Martins-Souza *et al*., 2011). Non-linear dose-response relationships were observed for all chemicals when applied to the encapsulation assay (Figure 4.4). The results of this assay indicates that the encapsulation response may be altered in the presence of acute exposure to the studied chemicals, although significant differences to the control (according to Dunnett's test) were only evident in the highest doses of BPA and DDE (2mg/L and 5mg/L respectively) (Figure 4.5). These are values which likely exceed environmentally 'realistic' levels in river water, although little is known about the concentrations of these chemicals in rivers that are needed to reach equivalent internal concentrations within the snail.

For the study of phagocytosis I investigated two approaches. One approach was relatively 'low-tech' but high-throughput (MTT assay) based on an adaptation of work by Bezerra *et al*., (1997). The second approach was essentially the opposite (high-tech, low-throughput) and is, to my knowledge, the first application of imaging flow-cytometry to molluscan cells. This approach was also supplemented by the development of a highly accurate counting mask for quantification purposes.

The relationships between the study chemicals and phagocytosis (measured as the mean number of beads within the cell) were similar to those from the encapsulation assay (Figure 5.11). This result was not unexpected due to the similarity of the mechanisms involved in encapsulation and phagocytosis. Indeed, the main difference

between these two approaches is size of the target and not the cell response (Bayne and Fryer, 1994). For this reason, the ERK pathway could be a potential target for future investigation of the studied chemicals. Indeed, ERK is a key pathway for these processes and has previously been shown to be affected by xenobiotic chemicals, at environmentally relevant concentrations in molluscan *in vitro* tests (Canesi *et al*., 2004). ERK (Extracellular-signal-regulated kinase) is a serine/threonine protein kinase and member of the mitogen- activated protein kinase (MAPK) family found in both vertebrates and invertebrates (Walker, 2006).

The imaging flow-cytometry assay was also applied, for the first time, to another mollusc species - *M. cornuarietis* (Chapter 5, Section 5.2.6.2). Although I did not investigate this snail species to the same level of detail as *B. glabrata*, I would strongly encourage further consideration of this species as a potential model for mollusc immunology studies based on the nature of the hemocytes (lack of clumping) and other advantageous physical attributes (size/hemolymph volume).

Of the *in vitro* assays, motility stood out as the endpoint most sensitive to the test chemicals, with strong negative relationships induced by most study chemicals (Figure 6.12). Interestingly, as for encapsulation and phagocytosis, ERK is also known to strongly regulate *B. glabrata* hemocyte motility (Zelck, 2007). Preliminary work would suggest that hemocytes remained viable over the dose-range of all the tested chemicals, so cell death was not believed to represent a significant factor.

The cell motility work was performed using a system which I developed in an attempt to merge low-cost and high-throughput data acquisition (Chapter 6, Section 6.2). While the microscope system was originally developed to aide my work on *B. glabrata* hemocyte motility, its potential for wider applications became quickly apparent. The system performed well with other, much more widely used cell types (mouse Sertoli cells and human breast cancer cells), and as a result it gained attention from numerous national and international media outlets, but more importantly from researchers in diverse fields from neurobiology to geology.

As with all immunotoxicological studies, alterations in the response of selected immune parameters may not have a direct bearing on immune competence or increased probability of infection or proliferation (Hoeger *et al*., 2004). For this reason, an *in vivo* assay was conducted in order to establish whether the *in vitro*

responses observed using the methods developed here could be observed in the intact snail. A UK effluent extract was used as the test chemical since this represented the most environmental relevant approach available.

After developmental exposure to effluent, snails were measured prior to infection. A negative linear relationship was evident suggesting decreasing size with increasing effluent concentration (Figure 7.8). While it is known that smaller snails are more susceptible to infection, internal parasite numbers were consistent among treatments (Table 7.9). Despite this, there was a strong tendency toward increased cercarial shedding with increasing effluent concentration (Figure 7.9), as well as increased evidence of toxic damage to the digestive tissue of the snail (Figure 7.20). These results seem to suggest an effect of effluent which enhances the ability of the cercariae to emerge. This effect is likely due to a direct effect on the snail rather than an enhancement of the parasite since parasites were not directly exposed to effluent, although I cannot rule out the possibility of parasites accumulating toxins stored in host tissue.

Having established methods during *the in vitro* phase of the project, I was able to employ the most cost/time-effective approaches to hemocytes derived from intact snails used in the *in vivo* assay. It appears that effluent exposure when coupled with infection had a synergistic effect toward lowering both phagocytosis and motility more than either factor alone. What is interesting to note is that one of the survival mechanisms used by *S.mansoni* whilst developing inside the snail is to secrete proteins that are known to disrupt ERK signalling (Bayne, 2009; Negrão-Corrêa *et al*., 2012) thereby effectively suppressing the immune response.

Given all the above, it is apparent that the pollutants used in this project can affect the interaction between *B. glabrata* and *S. mansoni* through possible suppression of immune responses. While this is an important conclusion, it leaves many questions. When we bring together all of the findings and put them into context with the literature, they do point to a possible explanation for the observed relationship between pollution levels and disease transmission.

In molluscan hemocytes ERK has previously been shown to be affected by xenobiotics, including those used in my studies (Canesi *et al*., 2004). ERK is known to be important in the processes of phagocytosis, encapsulation and motility in *B.*

glabrata hemocytes (Zelck, 2007; Toldedo and Fried, 2011). *S. mansoni* utilizes disruption of ERK to suppress the snail immune response and enhance its survival prospects (Bayne, 2009; Negrão-Corrêa et al., 2012). Reduction in phagocytosis is correlated with increased cercarial shedding (Connors *et al*., 1995).

In many ways thse observations would appear to represent an amalgamation of these previous findings, suggesting that down-regulation of hemocyte ERK as a result of combined pollution and infection results in decreased immune function and increased cercarial shedding.

Motility may be the most important factor under consideration since both the rate of encapsulation and phagocytosis is dependent on motility, but motility is not dependent on phagocytosis or encapsulation. The finding that motility was generally the most sensitive endpoint could be explained by the nature of the responses. While motility is an important factor in the other processes, even cells with reduced motility will likely be able to undergo a degree of phagocytoisis/encapsulation due to the fact that some cells will be closer to the foreign object (sporocyst, bead) than others and thus have to travel less distance. It may be that the effects I have observed are occurring on the motility process only and that this has a subsequent impact on the associated endpoints. Alternatively, it may be that a common mechanism affecting all measured endpoints, such as ERK, is affected. It is difficult to distinguish between these various possibilities due to the interactions between these endpoints which (in reality) could be seen as one continuous process but are typically studied in isolation.

It is important to note that outside of a laboratory setting, the parasite-host relationship is subject to many more variables, and with a much greater degree of variation, than chemical exposure alone. It is therefore not possible to make extensive conclusions regarding possible human health issues based on the nature of this kind of work and the literature, especially given its scarcity (Lefcort *et al*., 2002).

In general, this project represents a broad approach to a complex subject. The scarcity of existing research on this topic has meant that the project was less able to take advantage of well-established existing hypothesises and explore them in great detail, but instead it can be seen as a starting point for future investigations.

The nature of the project provided scope to explore some creative and novel approaches to the hypothesis, as opposed to saturated research fields where new experimental methods are developed by companies to enable researchers to produce more results. Consequently, much time was spent preparing the technical platforms needed to generate results and establish the theoretical basis for my hypothesis, which left little time to explore the mechanisms responsible in as much detail as I would have liked. For example, in order to answer some of the questions which have arisen from our results, it would have been extremely beneficial to investigate the parasite-host/effluent relationship at a molecular level. Molecular work focusing around signalling pathways, in particular effects on ERK signalling, would help to prove or disprove one of the main hypothesises to come from this project. Performing this kind of molecular work would be a logical progression to this research and would bring ecotoxicology and parasitology even closer together in a mechanistic sense.

Some of the main limitations with this project have been due to the logistical difficulties associated with handling hazardous effluent and potentially human-infective parasites. In general, ecotoxicology laboratories are set-up to deal with hazardous chemicals and parasitology laboratories are set-up to accommodate infectious samples. Since these two requirements are very different, I had to balance certain trade-offs in order to undertake these studies. For example, I would have liked to expose snails from eggs right through the infection process in a manner more representative of field conditions. Instead I had to settle for a developmental exposure in effluent, followed by infection of snails under clean water conditions. The consequence of this 'choice' on the research findings and their interpretations is presently unclear. However, it suggests that the parasites may have had a substantially reduced exposure to xenobiotics than the snails; something which perhaps would favour parasite proliferation/shedding as was observed. And so while we were able to provide answers to the main hypothesis (i.e. dose pollution affect parasite-host interactions?) the precise environmental relevance remains to be established. For this reason, another necessary extension of this work would be to perform the whole *in vivo* exposure again in the presence of xenobiotics. This would both repeat aspects of the *in vivo* exposure, and take the research forward.

Nevertheless, the *in vivo* study design was appropriate given the difficulties and risks posed by, for example, a flow-through system.

Due to the time constraints imposed by having to establish many of the assays form the beginning, or for their first use with hemocytes, as well as the difficult nature of *B.glabrata* hemocytes (clumping, low cell volume), I was unable to explore the responses of resistant snail strains. I would have liked to explore the question of whether resistant strains become *less resistant* when exposed to xenobiotics, and perhaps run parallel experiments against resistant and susceptible strains. Resistant strains (BS90) were extremely difficult to work with due to their smaller size, this was most apparent when large volumes of hemolymph were required for assays. Much work on resistant snails is of a molecular nature for which smaller volumes of hemolymph are likely to be sufficient, however they were much less suitable than susceptible snails for my work. The resistant snails also appeared far less hardy than their BBO2 counterparts, indeed we frequently lost snails and found it difficult to maintain a population. Superficially it appears as if resistance to *S.mansoni* imposes a cost on the general longevity of the snail, this is something which has previously been reported (Blair and Webster, 2007; Humphries 2010).

A further, and extremely important, extension would be to perform field work and/or epidemiological studies. For example, studies could be conducted which integrate information regarding human and snail parasite burdens along a transmission foci, with data regarding the composition and type of chemicals present in the water. Similar studies are already fairly routine for disease mapping purposes and I expect it would be relatively straightforward to marry this data with chemical analysis of water samples collected at the same time. Therefore, to summarise, my recommendations for future work would take two, almost opposite, paths. One would involve delving into greater detail in specific parts of the relationship i.e. molecular work, whereas the other would involve moving from the laboratory and attempting to understand the process at the highest and most environmentally relevant levels i.e. field studies and epidemiology.

8.1. Conclusion

In this project I have developed and adapted techniques to combine observations on the immune response and relationship between *B. glabrata* and *S. mansoni* before and after exposure to various xenobiotics and an effluent extract. The work described here represents a timely investigation into a potentially very important, yet understudied, area. My results suggest that globally distributed aquatic xenobiotics may act, in combination with *S. mansoni* infection, as immunosupressors, resulting in increased parasite shedding. I believe that my broad approach to investigate the effect of chemicals on immune parameters in *B. glabrata* could form the basis for more specific mechanistic investigations and to test my conclusions in real-world settings.

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