Final Report

Development of alternative approaches for monitoring the effects of the mosquito control agent Bti on ecosystems of the Dalälven catchment

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Utökad svensk sammanfattning, inklusiv ett preliminärt förslag till övervakning av Bti-effekter

Masskläckningar av översvämningsmyggor i området utmed Nedre Dalälven har en negativ påverkan på livskvaliteten för människor som bor i området. För att dämpa dessa årligen återkommande masskläckningar har man sedan flera år tillbaka använt det biologiska bekämpningsmedlet Bti. Bti utgörs av bakterier som dödar mygglarverna i översvämningsområden genom att angripa deras tarmepitel. Även icke-mål organismer, främst fjädermyggor (Chironomidae) och andra tvåvingar inom underordningen Nematocera, kan påverkas negativt av Bti-användningen. Fjädermyggor är en nyckelgrupp i akvatiska ekosystem och adulta fjädermyggor utgör även föda för många organismer på land. Effekter av Bti på fjädermyggor kan potentiellt ha stora konsekvenser för ekosystemen. I en studie i franska våtmarker har man t.ex. visat att en 66%-ig minskning i biomassan av myggor och fjädermyggor påverkar svalors diet och leder till minskad häckningsframgång.

NV har uppdragit till SLU att utreda pågående övervakning av effekter på miljön av Bti-användningen i Nedre Dalälven. Målet med forskningsutredningen är att:

> ”ta fram en rigorös och statistiskt fullgod uppföljningsdesign som mäter direkta effekter av bekämpning både på målorganismen, dvs översvämningsmygg (främst Aedes sticticus och A. vexans) och på andra icke-målorganismer såsom fjädermygg (chironomider). Fjädermyggen kan ses som en indikatorgrupp för att bedöma tillståndet hos andra evertebratgrupper. … Även näringstillståndet (övergödning) i vattnet har betydelse för myggens tillväxt, så en bedömning av bekämpningens effekt på näringsstatusen bör övervägas som del i programmet.”

Denna rapport beskriver ett miljöövervakningsprogram baserat på följande komponenter:

I. En litteratursammanställning som fokuserar på effekter av Bti-användning på icke-målorganismer och andra ekosystemkomponenter (t.ex. förändringar i näringsväven) och på miljöövervakningsansatser i Sverige och andra länder för att mäta effekter av Bti.

II. En översikt över de metoder som används för applicering av Bti och biologisk miljöövervakning av dess effekter hos myndigheter på andra håll i världen.

III. En utvärdering av den uppföljning av biologiska effekter av Bti så som den har skett i Sverige, inklusive dess utformning med avseende på provtagningsfrekvens, provstorlek och statistiska metoder.

IV. En analys av befintliga data från den biologiska uppföljningen av Bti-effekter, omfattande en utvärdering av den statistiska styrkan av provtagningsprogrammet, rekommendationer för förbättringar och förslag till alternativa analysmetoder.

V. En GIS-kartering av Nedre Dalälvens område med fokus på terrängformer och översvämningskänslighet.

VI. En sammanfattning av ett basalt biologisk miljöövervakningsprogram med en tillräcklig hög rumslig- och tidsmässig upplösning för att kunna utföra tillförlitliga, vetenskapliga bedömningar av Bti-effekter på stickmyggor och icke-målorganismer. Vidare ges förslag till utvidgning och förbättring av det basala programmet.

I) Litteratursammanställning – Sammanfattning av resultat

1. De Bti-doser som behövs för att orsaka dödlighet hos fjädermyggor överstiger vanligtvis de operationella doser som används för bekämpning av stickmyggor. Det finns dock i den vetenskapliga litteraturen också exempel på ökad dödlighet hos fjädermyggor vid operationella doser som använts vid Bti-användning.
2. Mångårig uppföljning av Bti-effekter i Nedre Dalälven har inte påvisat några effekter på icke-målorganismer (d.v.s. fjädermyggor).
3. Däremot har påtagligt negativa effekter av Bti-användning på tätheten och diversiteten av icke-målorganismer påvisats i biologiska miljöövervakningsprogram i Wales och Minnesota (USA). Även en studie från Frankrike visar på negativa effekter av Bti-användning på populationstätheten av evertebrat predatorer som spindlar och trollsländor och på fåglars häckningsframgång.
4. Motsägelsefulla resultat av Bti-effekter från olika långtidsstudier kan bero på olika biologiska och miljömässiga förutsättningar i de områden som har studerats. Så kan till exempel avsaknaden av tydliga effekter i området kring Nedre Dalälven bero på en hög andel ”terrestra” fjädermyggarter.
5. De motsägelsefulla resultaten i olika studier/utvärderingar kan dock också vara orsakade av skillnader i provtagningsmetoder och -strategi (d.v.s. provtagningsintensitet och replikering). Så har man till exempel vid ovan nämnda studie i Minnesota tillämpat en tre-gånger högre rumsmässig replikering, d.v.s. tagit

prov på tre gånger så många våtmarksområden, än vid den biologiska övervakningen som skett i området kring Nedre Dalälven.

6. Det är beaktansvärt att studier som rapporterar effekter av Bti på icke-målorganismer har provtagit fjädermygglarver i sjö/vårmarkssediment, medan man i kontrollprogrammet i Nedre Daläven har kvantifierat adulta fjädermyggor med hjälp av kläckningsfällor. Det är dock svårt att dra några generella slutsatser då antalet studier är lågt. Det känns dock angeläget att jämför fångsteffektiviteten av adulta fjädermyggor med hjälp av kläckningsfällor med den för metoder där bottenlevande larver provtas direkt i bottensedimentet.
7. Över det hela taget ger vår litteratursammanställning tydliga indikationer om att Bti kan ha både direkta negativa effekter på fjädermyggor och indirekta effekter på organismer högra upp i näringsväven. Dessa indikationer berättigar behovet av ett välutformat övervakningsprogram som på ett tillförlitligt sätt kan kvantifiera dessa effekter.

II) Biologisk övervakning av Bti-effekter i andra länder.

Endast två myndigheter svarade på vårt utskick med frågor kring ansatser för biologisk övervakning av Bti-effekter. I staden Winnipeg (Kanada) upplever myndigheter Bti-användningen som ytterst positiv. I Winnipeg mäter man för tillfället endast effekter av Bti-spridning på stickmyggor, ej eventuella effekter på icke-målorganismer. Organisationen *La Tour du Valet* i Frankrike har däremot ett övervakningsprogram som också omfattar icke-målorganismer, t. ex. trollsländor, fåglar och vattenväxter, och även näringsvävseffekter. Det mer omfattande franska uppföljningsprogrammet kan därmed fungera som förebild för ett övervakningsprogram som kan kvantifiera både direkta och indirekta effekter av Bti-spridningen.

III) Utvärdering av tidigare övervakning 2002-2007:

Under perioden 2002-2007 har CO2/ljusfällor och kläckningsfällor använts för provtagning av adulta mål- och icke-målorganismer. Programmet har haft god tidsmässig täckning (veckovis provtagning under översvämningsperioden), men mycket begränsad geografisk täckning, då endast fyra kläckningsfällor från vardera av sex översvämningsområden har använts. De sex översvämningsområdena har delats in i tre referensområden (ingen Bti-behandling under perioden 2002-2007) och tre behandlade områden (med minst 2-års Bti-behandling under perioden 2002-07), varav ett av sumpskogskaraktär med dominans av klibbal och två av ängskaraktär med starr och örtvegetation.

Ansatsen med kläckningsfällor för att kvantifiera icke-målorganismer som man har tillämpat i Nedre Dalälven har sina begränsningar, särskilt för organismer med en heterogen fördelning i terrängen. Så är till exempel fixerade kläckningsfällor inte lämpliga för att få ett representativt mått på stickmyggor, då dessa uppvisar en heterogen och fläckvis fördelning i den översvämmade terrängen. Även larver av akvatiska fjädermyggor uppvisar sannolikt en icke-homogen, fläckvis fördelning inom våtmarkerna (t.ex. koncentrerade till fuktiga/vattenfyllda depressioner i terrängen). Det är därför oklart om kläckningsfällorna utgör lämpliga redskap för att få ett representativt prov på samtliga viktiga icke-målorganismer, desto mer då endast fyra replikat per våtmarksområde har tillämpats.

Trots att de insamlade data från det nu operativa miljöövervakningsprogrammet är av multivariat karaktär, har statistiska analyser av data mestadels gjorts med “univariate” metoder. De statistiska modeller som redovisas i publikationer baserade på data från 2002–2007 är inte beskrivna i detalj och verkar inte ha utgått från en s.k. “Before-After Control-Impact” upplägg, som tillåter de tydligaste slutsatserna kring mänsklig påverkan på ekosystemen. De tillämpade statistiska modellerna kan också optimeras för att ta hänsyn till olika variationskällorna i datamaterialet.

IV) Statistisk analys av befintliga data

Vi har utfört statistisk analys av data från åren 2002–2007, som insamlats inom ramen för det befintliga biologiska uppföljningsprogrammet, med följande syften:

1. Att tillämpa bäst möjliga “Before-After Control-Impact” upplägg för analys av data med lämpliga variansanalysmodeller,
2. Att beräkna den statistiska styrkan för de variansanalysmodeller som använts i den befintliga uppföljningen av biologiska effekter av Bti-appliceringen, samt att beräkna vilken replikeringsnivå som skulle behövas, d.v.s. hur många prover som skulle behöva tas, för att uppnå en godtagbar statistisk styrka,
3. Att föreslå alternativa analytiska angreppssätt som kan appliceras på datasetet.

Observera att dessa syften INTE omfattar en omarbetning av befintliga analyser som redan har publicerats, då detta låg utanför ramen för detta uppdrag och skulle kräva en betydligt större förtrogenhet med de insamlade data än vad den relativt korta tidsperioden har tillåtit. Därför måste alla analyser i denna rapport betraktas som illustrerande i sin karaktär och kan INTE användas för att dra generella slutsatser kring effekter av Bti-behandling på olika organismgrupper.

Analys av statistisk styrka för den befintliga uppföljningen av biologiska effekter av Bti-appliceringen, analyserat med den mest lämpliga statistiska modellen, visar att uppföljningsprogrammet med godtagbar statistisk styrka kan detektera effekter av Bti på vissa variabler när dessa effekter är väldigt starka (t.ex, om Bti skulle orsaka en 66%-ig minskning i populationstäthet och diversitet av fjädermyggor). Det befintliga uppföljningsprogrammet uppvisar dock en mycket låg statistisk styrka för att upptäcka mindre effekter (t. ex. om Bti skulle orsaka en 10%-ig minskning i fjädermyggors populationstäthet). Avsevärt högre statistisk styrka kan åstadkommas genom (i) relativt måttliga ökningar i antalet replikata våtmarker (till mellan 10 och 16), (ii) stratifiering av olika miljöer, t.ex. torra (terrestra) och blöta (akvatiska) faser av våtmarkerna, samt (iii) en ökning av antalet replikat inom varje våtmarkstyp.

Multivariat analys, t.ex. ordinationstekniker eller likhetsanalys (Analysis of Similarities) och SIMPER, föreslås för alternativa angreppssätt för dataanalys.

V) GIS-analys av potentiella referensområden i Nedre Dalälven

Geografiska Informations System (GIS) har använts för att identifiera potentiella referensområden utanför de områden som fram till 2010 har behandlats med Bti. Dalälvens sjösystem uppdelades i 7 delar utifrån höjddata. För att ta fram områden som kan påverkas av översvämning gjordes en urval av områden med en höjd max 1 m över sjöytorna. Ett urval av områden med TWI (terrain wetness index) över 0 gjordes och kombinerades med de beräknade översvämningsområdena, och myrmarker från kartinformation. Ur detta material och med hjälp av tidigare provplatser gjordes ett urval av nya områden där övervakningsstationer skulle kunna läggas. Områdena jämfördes visuellt med Google Maps kartor som i området håller en hög kvalitet, och utifrån dessa kunde ett antal platser som även ligger lättillgängliga väljas ut. Utifrån flygbilderna i Google Maps kunde flera områden avfärdas och några läggas till som intressanta. På detta sätt har 20 olika lokaler/områden identifierats som kan fungera som referensområden, då de är känsliga för översvämningar (Appendix 3 och http://infogis.ma.slu.se/map/mygg/). Fler information om dessa lokaler kan erhållas genom kontakt med författarna.

VI) Preliminärt förslag till övervakning av Bti-effekter

(A) Överväganden

Nedre Dalälvens miljö, med återkommande vattenståndsvariationer och en mångfald av naturtyper kännetecknas av en mycket stor rumslig och tidsmässig variation. Den stora mellanårsvariationen i frekvensen och varaktigheten av översvämningstillfällen och temperaturförhållandena, i samband med en hög rumslig variation orsakad av mikrotopografi och vegetationsförhållanden, har stora effekter på utvecklingen och fördelningen av populationer av både mål- och icke-målorganismer. Denna stora variation utgör en utmaning för utformningen av biologiskt uppföljningsprogram. Till exempel, en lämplig tidpunkt för provtagning innan Bti-appliceringen kan varierar kraftigt mellan åren beroende på översvämningarnas tajmning och populationsutvecklingen av översvämningsmyggorna. På samma sätt kan en lämplig lokal för provtagning av akvatiska icke-målorganismer vara olämplig ett annat år, beroende på variation i storleken av den översvämmade området. I Nedre Dalälvens område utgörs icke-målorganismerna av både renodlat akvatiska arter och arter som lever i en terrester habitat. Båda dessa grupper kan förväntas uppvisa mycket olika responser på ett översvämningstillfälle och en behandling av terrängen med Bti. Terrestra fjädermyggor lever i mindre vattensamlingar i skogen eller är associerade med bestånd av kärrvegetation. Dessa terrestra arter exponeras därför sannolikt i mindre omfattning för Bti än arter i en renodlat akvatisk miljö.

För att statistiskt kunna säkerställa eventuella effekter av Bti med hänsyn till den störa variation i Nedre-Dalälven, föreslår vi att man i ett framtida övervakningsprogram (i) ökar antalet övervakade översvämningsområden och (ii) ökar antalet prov som tas inom varje översvämningsområde. Vidare förslår vi att man i ett framtida uppföljningsprogram tillämpar ett s.k. "*pooling och subsampling*" protokoll i provhanteringen. Detta innebär att samtliga replikat från ett översvämningsområde först slås samman (*poolas*), för att sedan analysera att antal slumpmässigt utvalda, kvantitativa delprover ("subsamples") tills ett förutbestämt antal individer, vanligen mellan 300-700, har sorterats ut från det sammanslagna provet. Ett sådant protokoll minskar kostnaden för provanalys, men bibehåller en god täckning av variationen inom varje översvämningsområde. Dylika ansatser är väl etablerade inom biologisk miljöövervakning.

För att ytterligare öka möjligheten att statistiskt kunna säkerställa en påverkan av Bti på ekosystemen rekommenderar vi att man i ett framtida övervakningsprogram (i) åtskiljer provtagning av de akvatiska och terrestra faserna av översvämningsområdena, (ii) stratifierar provtagningen för de två viktigaste typerna av översvämningsområden, d.v.s. sådana som är av sumpskogskaraktär med dominans av klibbal och sådana av ängskaraktär med starr och örtvegetation, samt (iii) inkluderar fysikaliska och vattenkemiska mätvariabler som kan ge stöd vid utvärderingen.

Lokaliseringen av flera lämpliga referensområden är viktig och bör prioriteras. Man måste också säkerställa att dessa referensområden förblir obehandlade i ett längre tidsperspektiv, dels för den långsiktiga miljöövervakningen och för studier av långsiktiga effekter av Bti på ekosystemen. Vidare föreslår vi att man överväger att ta ytterligare bottenfaunaprov, till exempel med rörhämtare, då denna metod har visat sig vara framgångsrik i andra länder för att upptäcka Bti effekter.

B) Preliminärt förslag och kostnadsuppskattning

Nedan redovisar vi två förslag till ett förbättrat framtida uppföljningsprogram för effekter av Bti, ett basprogram som endast täcker en typ av översvämningsområden och enbart akvatiska organismer, samt ett utökat program där båda dominerande typer av översvämningsområden, samt kompletterande provtagning av bottenfauna och terrestra fjädermyggor ingår. Kostnadsuppskattningen för båda förslagen nedan är baserad på kostnaderna per prov för den nationella miljöövervakning som SLU bedriver i uppdrag av NV.

1) Basprogram – Provtagning görs endast i översvämmade områden (> 1 cm ytvatten) och bara i behandlade översvämningsområden av ängskaraktär. Minst 6 obehandlade referensområden och 6-10 behandlade områden ingår.

- *Icke-mälorganismer* provtas med 10 kläckfällor per område och provtagningsdatum. Varje fälla exponeras i 48 timmar. Fällorna omplaceras slumpmässigt varje vecka under den mest dynamisk perioden (6-8 veckor när vattennivån förändras fort), annars varannan vecka. Inledningsvis ska fällorna placeras över hela det översvämmade området, för att sedan placeras över kvarvarande vattensamlingar när vattenståndet är lägre.

- *Målorganismer* provtas med 10 ljusfällor per område, installerade samtidigt som kläckfällorna och slumpmässigt fördelade över områdena. Varje fälla exponeras i 48 timm.
- *Fysikalisk-kemiska variabler* – Vattenprover (5 per område) tas på varje kläckprovningsdatum och analyseras med avseende på närsalter och andra basvariabler. Temperatur loggers (3-5 per våtmark) kan lämpligen appliceras under utvalda kläckningsfälla. Vattendjup intill varje kläckfälla mäts vid varje tömning.

Preliminär kostnadsuppskattning för basprogrammet (12 områden, 12 provdatum) landar på en årlig kostnad på 1,4 miljoner kronor. Av detta går ca 15% till fältarbete, 15% till utrustning, 50% till provbehandling och 20% till vattenkemiska analyser.

2) Utökat program – Provtagning omfattar förutom det som beskrivs i basprogrammet ovan även (i) översvämningsområden som är av sumpskogskaraktär (3-5 behandlade och referensområden, beroende på tillgänglighet), (ii) ytterligare kläckfällor för att provta terrestra fjädermyggor som förekommer i fuktig mark (ytvatten <1cm), samt (iii) bottenfaunaprov tagna med rörhämtare, med sammanslagning och uppdelning av replikat, såsom i bearbetning av prover från kläcknings- och ljusfällor.

Preliminär kostnadsuppskattning för det utökade programmet (20 områden, 12 prov per datum) landar på en årlig kostnad på mellan 2,0 (utan bottenfaunaprovtagning) och 2,5 miljoner.

C) Kommentarer – Förslaget måste betraktas som preliminärt. Det finns ett stort behov av en djupare analys av befintliga data, till exempel för att optimera antalet stickprov från varje område som behövs för en god statistisk styrka och för att kvantifiera antalet individer som behöver sorteras från de sammanslagna delproverna. Dessutom saknas grundläggande information om responsen från icke-målorganismer på översvämningar, om den småskaliga fördelningen av icke-målorganismer, samt om hur näring som härstammar från det stora antalet döda stickmyggor och majskornen som följer med Bti-appliceringen påverkar ekosystemen. Ett nytt övervakningsprogram kommer därför att initialt behöva genomgå en optimeringsfas. Andra, mer komplexa, frågor besvaras lämpligen av olika forskningsinsatser knuten till Nedre Dalälvsområdet.

Expanded English summary, including a preliminary suggestion for a Bti biomonitoring scheme

The mass emergence of bloodsucking mosquitoes in the lower Dalälven catchment seriously compromises the quality of life for inhabitants of the region. In recognition of this, the mosquito biocontrol agent Bti has been used during years of severe mosquito outbreaks. Bti is a bacterial biocontrol agent that is ingested by mosquito larvae, killing them *en masse* and effectively reducing adult numbers. Non-target organism (NTO) groups showing some vulnerability to Bti are, like mosquitoes, most likely to be members of the Diptera suborder Nematocera, particularly non-biting midges (Chironomidae, Swedish "fjädermyggor. Chironomids are a key component of food webs in both aquatic and terrestrial environments. However, even when Bti has minimal direct effects on NTO, it may potentially be associated with knock-on effects on other ecosystem properties. For example, 90-100% reductions in the biomass of adult mosquitoes may remove an important food source for terrestrial organisms. Indeed, recent evidence from a longer-term study indicate that 66% reductions in the biomass of emerging nematoceran dipterans (mosquitoes and chironomids) from wetlands treated with Bti alters the diet of birds, in turn affecting their breeding success.

In recognition of the ongoing need for mosquito control in Dalälven, the Swedish Environmental Protection Agency commissioned the Department of Aquatic Sciences and Assessment (DASA), Sveriges Lantbruksuniversitet (SLU) with the task of developing a scheme for monitoring the effects of Bti use. The commission included the development of a rigorous and statistically sound sampling design capable of measuring the effects of Bti use on both target and non-target organisms, and which also takes consideration to additional ecosystem effects, including on ecosystem nutrient status.

This report delivers a description of a biomonitoring scheme, built on the following components:

I) A literature review focusing on the effects of Bti on non-target organisms (NTO) and other ecosystem properties (e.g. changes in food web characteristics), and on the approaches taken to biomonitoring the effects of Bti in Sweden and elsewhere.

II) A survey of the Bti treatment and biomonitoring methodologies employed by agencies elsewhere in the world.

III) An evaluation of the previous biomonitoring scheme, with consideration of the strengths and weaknesses of its sampling design, and its statistical methodologies

IV) An analysis of data collected by the previous biomonitoring scheme, with an evaluation of the statistical power of the previous sampling design, recommendations for improving power, and a demonstration of some alternative analytical approaches.

V) Mapping of the Dalälven catchment using Geographic Information Systems analysis, with a focus on landforms and flooding sensitivity.

VI) A summary of a basic biomonitoring scheme, which has a spatio-temporal scope that is sufficient for a reliable, scientific assessment of the effects of Bti on target and non-target organisms. Additional suggestions are given for expansion of the basic scheme.

I) Literature review: Summary of findings

1) Dosages of Bti required to induce mortality in Chironomidae are much greater than "operational" doses used in mosquito control, however there are examples of chironomid species that suffer mortality at operational doses also.
2) Long-term monitoring of the mosquito control program on the River Dalälven, Sweden, has not found any major effects of the use of Bti on NTO chironomids.
3) In contrast, monitoring of mosquito control programs in Wales and Minnesota, USA, have revealed often marked effects on the density and diversity of NTO, and in France there is evidence that the breeding success of birds and abundances of intermediate predators (spiders and dragonflies) are reduced in areas treated with Bti.
4) Contrasting results from different long-term studies may relate to differences in local environmental and biological characteristics. For example, it is possible that the lack of strong effects observed in the Dalälven might reflect the high proportion of terrestrial chironomids in that system.
5) However, the contrasting results from the long-term studies might also reflect differences in sampling design and protocols. For example, the Minnesota study includes three times more spatial replication at the wetland scale than the Dalälven study
6) It is notable that all studies reporting effects of BTI on NTO have sampled chironomids in the larval stage from the benthos, whereas the Swedish Dalälven research program has relied on emergence traps, to sample emerging adult insects. Caution is needed in generalising from this observation, due to the low number of studies overall. Nevertheless, the effectiveness of the emergence traps for sampling the chironomids relative to benthic methodologies requires further assessment.

7) Overall, the literature review provides sufficient evidence of the potential for Bti to affect a major group of NTO, the Chironomids, and to have knock-on effects on other organisms mediated through changes in food web structure, to justify the need for ongoing monitoring of its effects.

II) Survey of biomonitoring in other countries

Only two agencies responded to our survey of biomonitoring approaches used in other countries. . The City of Winnipeg (Canada) regards the move towards Bti, a biocontrol agent, as overwhelmingly positive. Winnipeg have not instituted efforts to monitor the effects of Bti beyond those on the target organisms. In contrast, the French program coordinated by *La Tour du Valet* has taken a comprehensive approach to monitoring the NTO and other foodweb effects of Bti, and provides a potential model for the range of impacts that might be assessed.

III) Evaluation of the previous monitoring scheme

Published data from the previous biomonitoring scheme covers the period 2002-2007. Previous biomonitoring of both target (*Aedes stictatus*) and various non-target (Chironomidae, other macroinvertebrates, protozoa) organisms has focused on the same six wetlands, three treated with Bti in at least some years, and three reference wetlands not treated with Bti during 2002-07. Target species (nuisance mosquitoes) were mainly sampled using 3 light/CO2 traps per locale, trapping monthly for two nights June-August. NTO insects with a flying adult stage (predominantly chironomids) were sampled using modified “Mundie’s” emergence traps, left on the wetlands during the monitoring period (floating on the water during the aquatic phase, resting on the ground during the terrestrial phase). These were emptied weekly May to September. Originally 10 such traps were placed out per wetland, but only four were sorted on each sampling date, due to resource limitations. The four sites were chosen to represent a range of environmental conditions. The previous program thus had very good temporal coverage, with weekly sampling during the peak period for mosquito and chironomid emergence, and coverage of both the wet and dry phases of the wetlands. However, the program had very limited spatial coverage, both at the whole-wetland scale, with only 3 treated and 3 reference wetlands, and at the within-wetland scale, with only four emergence traps sorted per site.

The emergence trap sampling method used for Insect NTOs, while flexible, also has limitations, particularly with respect to sampling patchily distributed organisms. For example, fixed emergence traps would be unsuitable for sampling mosquitoes, due to the extremely patchy distribution of larval mosquitoes within the flooded wetlands. The distributions of some NTOs, such as the truly aquatic chironomids, may also be very patchy within wetlands. It is thus unclear whether the emergence trap method is suitable for the representative sampling of all important NTOs, particularly with only four replicates per wetland.

Statistical analyses of data from the previous biomonitoring program shows an over-reliance on univariate approaches for data that is inherently multivariate. The statistical models presented in publications from the previous program are not well-described, but appear not to have employed state of the art "Before-After Control-Impact" analyses that allow the strongest inferences on the effects of human intervention on ecosystem properties. The models could also be better optimized to account for different sources of variation in the data.

IV) Statistical analyses of existing data

We have carried out statistical analyses of data collected as part of the previous biomonitoring program from 2002-07, to address the following general aims:

1) To apply a best practice "Before-After Control-Impact" (BACI) design to ANOVA models for analyzing the data
2) To assess the statistical power of those models at the actual levels of replication used in the previous monitoring program, and to analyze the levels of replication required to increase statistical power to reasonable levels
3) To demonstrate some alternative designs and analytical techniques that could be applied to the data.

Note that these aims do NOT include the reworking of existing analyses, to replace those already published. This was beyond our terms of reference for this report, and would require a much deeper familiarity with the data than we could develop within a short time period. Thus, ALL analyses presented here are to be considered *primarily demonstrative in character*, and are *NOT intended for drawing general inferences* on the effects of Bti treatment or any other variable.

The power analyses demonstrate that the current biomonitoring scheme, analysed using the best possible statistical model, is capable of detecting very strong impacts of Bti on some ecosystem properties (e.g if Bti was to cause a 66% reduction in total chironomid abundance and diversity), but has very little power to detect more subtle effects (eg: 10% reductions in chironomid abundance and diversity). Substantial improvements in statistical power can be achieved through a relatively modest increase in the number of wetlands sampled (between 10-16), explicit separation of sampling of different environments (eg. aquatic vs terrestrial phases of the wetlands), and increasing the number of within-wetland replicates.

Multivariate analyses (an ordination, and analysis of similarities followed by SIMPER) are presented to demonstrate alternative techniques that could be applied to the data

V) GIS analysis of potential reference sites

Geographic Information Systems (GIS) analysis was used to identify potential reference sites outside of the areas treated by Bti during 2010, based on their likely sensitivity to flooding. The lakes in Dalälven catchment were first divided into 7 groups based on topogrophic information. To estimate the catchment areas sensitive to flooding, areas were selected based on their topographic height above the lake surfaces and based on the extent of mires recorded in maps. Specifically, sites were selected that were less than 1m above lake surfaces, and which had a TWI (terrain wetness index) above zero. These areas were compared visually with Google Maps, which have a high resolution in the Dalälven region, and potential biomonitoring sites with good accessibility were identified. Further potential sites of interest were chosen based on aerial photographs in the Google Maps Database. Based on these methodolgies, up to 20 potential sites were identified, based on their likely sensitivity to flooding (Appendix 3, and http://infogis.ma.slu.se/map/mygg/). Coordinates for these sites are available on request.

VI) Preliminary suggestion for the biomonitoring of the effects of Bti in the lower Dalälven

The development of a biomonitoring program for the use of Bti in Dalälven is complicated by the extreme variability characterizing its floodplain wetland systems. Inter-annual variability in the extant and duration of flooding and in temperature characteristics, along with spatial variation among and between wetlands in microtopography and vegetation characteristics, all have strong influences on the development and distribution of populations of both target (mosquitoes) and NTOs. This variation poses substantial challenges for planning biomonitoring, as an appropriate time for commencing before-treatment measurements will be dictated by the application-data of Bti, which can vary among years according to the timing of flooding and the mosquito development rates. Similarly, a suitable locale for sampling aquatic NTOs one year may be unsuitable the following. At Dalälven in particular, the NTO (chironomid) assemblage includes species ranging from fully aquatic to terrestrial taxa. These may be expected to respond differently to the extent of flooding, and to the application of Bti. Terrestrial chironomids live in water logged soils or associated with moist vegetation, and a priori might be expected to feed less on Bti delivered in a form designed for consumption in the aquatic environment.

The suggested biomonitoring scheme includes guidelines for two alternative monitoring programs: a base program and an expanded program. Both programs involve an expansion in the numbers of wetland replicates, the explicit separation of sampling of the aquatic and terrestrial phases of the wetlands, and the use of pooling and subsampling of replicates within wetlands to save sorting and identification-related labour costs.

The *base program* would be carried out only in flooded wetlands, and only in wetlands of the "wet meadow" vegetation type. Originally it was suggested that the base program should include at least 5 each of reference and treated wetlands, but following our power analyses, we now suggest increasing this number to at least 6, for 12 wetlands total, and preferably more. Other guidelines for the base program include:

- *Non target organisms* would be sampled using 10 emergence traps per site, sampling inundated habitats only (>1cm surface water). Each trap should be deployed for 48 hours and then removed. The traps are randomly redeployed weekly during the most dynamic period (6-8 weeks peak inundation), otherwise biweekly, initially covering the inundation floodplain, then with a focus on remaining water pools as the water levels recede.
- *Target orgnisms* would be sampled using ten combination CO_2-light traps per wetland, deployed randomly over the wetland for 48 hours and then removed, with random redeployment weekly during the active treatment period, otherwise biweekly.
- *Supporting Data* – Physico-chemical data: temperature loggers (3-5 per wetland), water nutrients (5 samples per wetland) and water depth (adjacent to each emergence trap) should be sampled from every wetland at every emergence trap clearance date.

Additionally, we now suggest that during periods of Bti treatment, water samples quantifying the effective dosages of Bti also be collected, if possible

A “pooling and subsampling” protocol will be used in all sample processing, whereby within-weland replicates are pooled, and then quantitative subsamples are randomly selected for further processing and identification, until a set number of individuals (typically between 3-700) are processed.

The expanded program would i) Add additional wetlands characterized by alder swamp vegetation (3-5 treated and reference, depending on availability), (ii) Add additional emergence traps sampling chironomids of the terrestrial phase (surface water <1cm), (iii) add benthic core sampling, with pooling and subsampling of replicates during processing.

In all cases, we recommend the use of both univariate and multivariate statistical approaches, with care taken to use statistical models that are replicated at the appropriate spatio-temporal scales, and account for multiple sources of variation in the data.

A preliminary cost estimation for the base program is 1,4 million Swedish Crowns, of which 15% is allocated to fieldwork, 15% to equipment, 50% to sample processing and 20% to chemical analyses.

A preliminary cost estimation for the base program falls between 2,0 (without extra bottom fauna samples) and 2.5 million Swedish Crowns.

(1) Preamble: Background and scope of this report

The mass emergence of bloodsucking mosquitoes in the lower Dalälven catchment seriously compromises the quality of life for inhabitants of the region[1]. In recognition of this, the mosquito biocontrol agent Bti has been used during years of severe mosquito outbreaks. Bti is a bacterial biocontrol agent that is ingested by mosquito larvae, killing them *en masse* and effectively reducing adult numbers. However, its use in the Dalälven is problematical on several grounds, including (i) the Natura 2000 status of large sections of the catchment, (ii) the use of Bti in national parks and other protected areas within the catchment, (iii) the large scale application of Bti, (iv) potential effects on non-target aquatic organisms and (v) potential whole ecosystem effects, which are unlikely to be limited to aquatic environments, but may also include effects on terrestrial organisms (eg. birds) that depend on emerging aquatic insects for food[2].

During 2009, Nilsson and Renöfält (2009)[3] evaluated the existing biomonitoring of Bti in the Dalälven catchment. While emphasizing that the use of Bti should continue for humanitarian reasons, the report was critical of the limited biomonitoring that had been undertaken to that point. In particular, the limited spatial and temporal scope of the monitoring and lack of good control data was highlighted, as was its failure to consider ecosystem effects beyond those on the target organisms (mosquitoes) and other closely related species.

Nilsson and Renöfält (2009)[3] made a number of recommendations, emphasizing the need for a long term monitoring research program, preferabluy driven by independent (ie: with no commercial interest in Bti) researchers and evaluating a wide range of possible effects of Bti, both positive and negative. In particular, they highlighted the need to not only consider the dose-responses of target and non-target organisms to Bti, but also effects on aquatic and terrestrial food webs that could arise from, for example, the accumulation of a large biomass of rotting mosquito corpses in the aquatic environment, and a reduction in the biomass of emerging insects important for birdlife and other terrestrial organisms in the region. The necessity of good control sites and extensive spatio-temporal coverage was also emphasized.

In recognition of the ongoing need for mosquito control in Dalälven, our institution, the Department of Aquatic Sciences and Assessment (DASA), Sveriges Lantbruksuniversitet (SLU) was given the task of developing a scheme for monitoring the effects of Bti use. DASA has a long history of developing and executing the scientific assessment of a diverse array of human influences on aquatic ecosystems in Sweden, and coordinates a number of current biomonitoring programs.[4] This report delivers a description of a biomonitoring scheme, built on the following components:

I) LITERATURE REVIEW

- A literature review focusing on the effects of Bti on non-target organisms (NTO) and other ecosystem properties (e.g. changes in food web characteristics), and on the approaches taken to biomonitoring the effects of Bti in Sweden and elsewhere. The review is delivered in full in Appendix I.

II) SURVEY OF AGENCIES

- A survey of the Bti treatment and biomonitoring methodologies employed by agencies elsewhere in the world. Survey responses are given in full in Appendix II.

III) EVALUATION OF THE PREVIOUS BIOMONITORING SCHEME

- An evaluation of the previous biomonitoring scheme, with consideration of the strengths and weaknesses of its sampling design, and its statistical methodologies

0) ANALYSIS OF EXISTING DATA

- An analysis of data collected by the previous biomonitoring scheme, with an evaluation of the statistical power of the previous sampling design, recommendations for improving power, and a demonstration of some alternative analytical approaches.

V) GIS MAPPING OF DALÄLVEN AND RECONNAISSANCE:

- To assist in the identification of possible control sites, the Dalälven catchment has been mapped using Geographic Information Systems analysis, with a focus on landforms and flooding sensitivity. Some potential reference sites have been visited. The GIS map is delivered in Appendix III, and via weblink.

VI) DEVELOPMENT AND DESCRIPTION OF A BIOMONITORING SCHEME

- A summary of the proposed biomonitoring scheme has been prepared in Swedish and includes an economic analysis. The biomonitoring scheme was developed with consideration of the findings of the preceding project components I-V. The emphasis was on developing a basic scheme with a spatio-temporal scope that is sufficient for a reliable, scientific assessment of the effects of Bti on target and non-target organisms. Additional suggestions are given for expansion of the basic scheme. The program summary is given in Appendix IV.

Following a brief introduction, this report is divided into sections addressing these components in turn.

(2) General Introduction: Bti, non-target organisms, and biomonitoring

2.1 The use of Bti: effects on target and non-target organisms

The bacterium *Bacillus thuringiensis serovar israelensis* (Bti) was isolated from a stagnant pond located in the Negev in south Israel in 1977[5]. Subsequently, Bti has been proven in lab experiments and field research to be a highly targeted control agent for biting insects from the dipteran (true fly) suborder Nematocera, especially mosquitoes Culcidae) and blackflies (Simuliidae), with very few documented non-target organism (NTO) effects[5,6]. Bti produces crystal aggregations containing multiple toxins that disrupt the gut wall of target organisms[7-9]. Today, Bti is commercially produced in various formulations for use all over the world in the control of nuisance mosquitoes and black flies.

During more than 30 years of commercial use, the effectiveness of Bti as a mosquito contol agent has been extensively evaluated. Bti typically causes 90-100% mortality of target organism larvae, while largely harmless for most other aquatic and terrestrial species[5,10,11]. NTO organism groups showing some vulnerability to Bti are, like mosquitoes, most likely to be members of the Diptera suborder Nematocera. In particular, non-biting midges (Chironomidae, Swedish “fjädermyggor”) may be vulnerable to Bti[6,12]. Chironomids are a key component of food webs in both aquatic and terrestrial environments[13]. The family is species-rich (10 000+ species world wide) and ecologically diverse, with larvae occurring in fresh and salt water and on land across all altitudes and most latitudes, including Antarctic regions[13]. Apart from chironomids, there are almost no reliable observations of negative effects of Bti on other NTO in either lake or stream ecosystems (see Appendix I, this report).

The results of toxicity studies in both laboratory and field conditions demonstrate that chironomids can be affected by Bti. However, the dosages required are much greater than for mosquitoes. For example, Bti has been documented causing 40-50% mortality among chironomids, but at dosages more than ten times greater than the “operational” dosages required for mosquito control[14]. Nevertheless, there is substantial variation in vulnerability within the Chironomidae, and some species are affected even at operational dosages[5,15].

It is likely that variation in the vulnerability to Bti among chironomid species relates at least in part to differences in their ecology. This is of particular importance at Dalälven, where the Chironomid assemblage is notable for a high diversity of terrestrial species[16,17]. These species live in moist soils or associated with damp vegetation[13], and their likely vulnerability to Bti preparations intended for consumption by larvae with aquatic feeding modes is not known.

Even when Bti has minimal direct effects on NTO, it may potentially be associated with knock-on, partially cascading, effects on other ecosystem properties, particularly food-web properties. Results from recent long term studies highlight some potential concerns surrounding the sustained use of Bti for aquatic and adjacent terrestrial environments, at least in some circumstances[2,18,19]. For example, 90-100% reductions in the biomass of adult mosquitoes may remove an important food source for terrestrial organisms. Indeed, recent evidence from a longer-term study indicate that 66% reductions in the biomass of emerging nematoceran dipterans (mosquitoes and chironomids) from wetlands treated with Bti alters the diet of birds, in turn affecting their breeding success[2]. This may partly reflect reductions in availability of polyunsaturated fatty acids, which are synthesized in aquatic ecosystems and carried into terrestrial environments by emerging aquatic insects, and which are important for the growth and reproduction of aquatic and terrestrial organisms[20]. More generally, the potential for smaller effects, including sublethal effects, of Bti on the biomass and diversity of NTOs to accumulate under sustained Bti use over several years is unclear.

The use of Bti should be accompanied by reliable biomonitoring of its effects on both target and NTOs, particularly in cases where it is applied over many years. Additionally, given that Bti removes a large quantity of insect biomass from the terrestrial food web, research should also be conducted to address potential effects on terrestrial ecosystems.

2.2 Biomonitoring in wetland ecosystems subjected to irregular flooding.

The mass-emergence of biting mosquitoes often occurs in association with the inundation of flood-plain wetlands, as is the case in the lower Dalälven, Sweden[1]. Such flood events are associated with a high degree of hydrological and biological variability, over multiple spatio-temporal scales[1,16]. Between years, floods vary greatly in their occurrence, ranging from dry years without flooding, to wetter years with extensive inundation. Within wet years, floods may vary in their timing (spring vs early-mid summer), and in the time it takes for them to recede. The impact of floods within individual wetlands can further vary according to local soil, vegetation and topographical characteristics. A floodplain that is topographically lowered relative to river banks and which lacks woody vegetation is likely to experience longer periods of inundation than higher floodplains characterized by mounds rather than depressions, and with a better cover of wood vegetation. The variability characterizing wetland ecosystems poses great challenges both for maintaining a mosquito control program, and for biomonitoring and research on the effects of Bti on NTO's and other ecosystem properties, and is addressed in the development of the monitoring program (Section 8)

(3) Summary of the literature review

The literature review, entitled "Assessing the effects of the mosquito control agent Bti (*Bacillus thuringiensis serovar israelensis*): a literature review of Swedish and international studies", is attached in Appendix I. The major findings of this review are summarized here, see Appendix I for the full reference list:

8) Bti is a highly specific biocontrol agent, with by far the strongest toxic effects on its targeted organisms: biting flies (Diptera) from the suborder Nematocera (especially mosquitoes and blackflies)[5]
9) Whilst Bti spores typically remain in treated environments after mosquito control operations, there is almost no evidence for any longer term residual toxicity associated with these spores, affecting either target or NTOs.[5,21]
10) Concerns have been expressed in the literature about the development of resistance to Bti in target organism populations repeatedly exposed to Bti treatment[22]
11) Few other organisms suffer direct toxic effects from Bti, the one major exception being members of the nematoceran subfamily Chironomidae.
12) Generally, the dosages of Bti required to induce mortality in Chironomidae are much greater than typical "operational" doses used in mosquito control, however there are examples of chironomid species that suffer mortality at operational doses also[5,15].
13) This implies that Bti has potential to alter the structure and diversity of chironomid communities, particularly if effects of repeated Bti exposures accumulate over time
14) Even if Bti is not associated with extensive impacts on NTOs, it might still affect the functioning of both aquatic and terrestrial environments by reducing the biomass of mosquitoes, an important food source for terrestrial and aquatic consumers, including birds. Additional effects on chironomid NTO would further confound such effects[2].
15) Long-term (>2 years) studies, necessary for detecting the cumulative effects of repeated Bti treatments on NTO chironomids, and knock-on effects on other ecosystem properties, are very few, and have yielded contrasting results.
16) Long-term monitoring of the mosquito control program on the River Dalälven, Sweden, has not found any major effects of the use of Bti on NTO chironomids[17].
17) In contrast, monitoring of mosquito control programs in Wales[23] and Minnesota, USA[18], have revealed often marked effects on the density and diversity of NTO, and in France there is evidence that the breeding success of birds is reduced in areas treated with Bti, associated with reductions in nematoceran dipterans in their diets[2].

18) There are also few assessments of knock-on effects of Bti use mediated through changes in food-web dynamics[19,24]. The most striking example is evidence for reduced breeding success of birds in French wetlands treated with Bti[2]. A newer publication from this program reports on further negative effects on intermediate invertebrate predators (spiders and dragonflies) associated with reed habitats[25].

19) Additionally, there is evidence that Bti is associated with altered productivity in certain sections of the aquatic assemblage, including reduced algal production[26], and in the Swedish Dalälven study, increased protozoan densities[27]. Further research is needed to assess the generality and ecological implications of these findings.

20) Contrasting results from different long-term studies may relate to differences in local environmental and biological characteristics. For example, it is possible that the lack of strong effects observed in the Dalälven might reflect the high proportion of terrestrial chironomids in that system. Differences in the susceptibilities of terrestrial versus aquatic taxa certainly needs more research.

21) However, the contrasting results from the long-term studies might also reflect differences in sampling design and protocols. For example, the Minnesota study includes three times more spatial replication at the wetland scale than the Dalälven study, and also extensive sampling prior to the Bti application[18]. This allows use of robust "Before-After Control-Impact" statistical models in analyzing the data. Bti was also applied more often during the Minnesota study, though inconsistency in reporting of dosages complicates direct dosage comparisons used in different studies

22) The French study is remarkable for the extent of additional monitoring undertaken, not only of NTO potentially affected directly by Bti, but also other organisms and ecosystem properties that might be affected through altered food-web structures[2,25].

23) It is notable that all studies reporting effects of BTI on NTO have sampled chironomids in the larval stage from the benthos, whereas the Swedish Dalälven research program has relied on emergence traps, to sample emerging adult insects. Caution is needed in generalising from this observation, due to the low number of studies overall. Nevertheless, the effectiveness of the emergence traps for sampling the chironomids relative to benthic methodologies requires further assessment.

24) Overall, the literature review provides sufficient evidence of the potential for Bti to affect a major group of NTO, the Chironomids, and to have knock-on effects on other organisms mediated through changes in food web structure, to justify the need for ongoing monitoring of its effects.

(4) Results from a survey of Bti management and monitoring practices in other countries.

A set of questions (Appendix II) were sent out to four agencies in four countries involved in the monitoring and management of mosquito control programs using Bti:

1) The American Mosquito Control Association,
2) The German agency *Bundesamt für Umweltschutz*,
3) The French agency *La Tour du Valet*,
4) Winnipeg City Council, Canada.

Of these, only the last two have provided detailed answers to the questionnaire. *Bundesamt für Umweltschutz* did not respond to two follow-up enquiries, while the American Mosquito Control Association indicated they would consult with multiple agencies to assist in answering the questionnaire, but had not responded at the time of preparation of this report.

The responses of the French and Canadian agencies are provided in full in Appendix II. Key points include:

1) Winnipeg treats mostly floodplain wetlands with Bti, during a program running from mid-April to September each year, while the French program targets mainly salt marshes in the Mediterranean south-east of the country, with 30-50 aerial treatments annually, and an unknown number of ground treatments
2) Winnipeg uses both granular and liquid formulations of Bti, while France uses liquid formulations
3) Both organizations monitor the effects of Bti on the main target organisms (biting mosquitoes)
4) Winnipeg does not have any monitoring of non-target organisms, stating: "We are aware that other Dipterans like the gnats are affected by Bti but with the narrow range of affected non-targets, the City of Winnipeg is increasing its use of Bti and other bacterial based products over the next few years to become fully biological with Bti being the primary control product"
5) The French agency , *La Tour du Valet* coordinates a monitoring program worth €120 000, focusing not only on the direct effects of Bti on NTO such as chironomids, but also on several other ecosystem properties. Topics covered include:
    a. Direct perturbations caused by mosquito-control operation in a natural reserve open to the public on human activities, resting ducks, tree-nesting herons, etc),

   b. Relative abundance of Odonata along observation transect,
   c. Density of chironomids and algae in marshes,
   d. Relative abundance of reed-dewlling invertebrates,
   e. Foraging activity and breeding success of bats and birds (house martin).

6) Most of the French monitoring activities are based on the comparison of 2-6 treated sites with untreated sites, and with sampling frequencies adapted to the phenology of the habitats/species studied.
7) Control sites in the French program were chosen based on their ecological similarity with treated sites. They have stated that in their case “the limiting factor is the number of treated sites since only 1/10 of the mosquito-producing habitats are actually treated.”
8) The French program incorporates before and after monitoring, especially for the chironomid and algal components
9) The Winnipeg program monitoring Bti effects on the target organisms also includes untreated control sites outside and within their mosquito abatement district (MAD), to test the effectiveness of Bti on various instars of mosquito larva. The sites are selected based on the same types of characteristics that occur within the MAD, including sites characterized by open, grassy vegetation, and by shaded, bushy vegetation, and covering sites with both high nutrient and low nutrient loads, and permanent, semi permanent and ephemeral systems. The Winnipeg program also includes pre- and post-treatment samples
10) Both agencies reported a high degree of public engagement in the program.
11) Specifically, the City of Winnipeg reported: “The public is extremely supportive of the movement towards the increased use of Bti in so far that the City of Winnipeg has budgeted for a completely biological program with Bti being the primary control product being used (planned for the season in 2012). We will still maintain alternative adult nuisance mosquito control with permethrins and adult control with Malathion 95 ULV”
12) *La Tour du Valet* reported: “The monitoring also involve a sociological study with interviews. People feelings are mixed, they would like to get rid of mosquitoes in inhabitated-areas without impacting nature”. And elsewhere: “Locals are happy with a strongly reduced nuisance”.

Assessment: The two programs that responded to our questionnaire provide strongly contrasting approaches to the monitoring of the ecological effects of Bti. The City of Winnipeg appears to have had a long history of chemical treatment of nuisance mosquitoes, and thus regards the move towards Bti, a biocontrol agent, as overwhelmingly positive. Winnipeg have not instituted efforts to monitor the effects of Bti beyond those on the target organisms. In contrast, the French program coordinated by *La Tour du Valet* has taken a comprehensive approach to monitoring the NTO and other foodweb effects of Bti. Both programs have emphasized the selection of extensive control sites and collection of data before and after treatment, while the French program provides a potential model for the range of impacts that might be assessed.

(5) Assessment of the current monitoring program

5.1 Monitoring Objects

Previous biomonitoring of both target (*Aedes stictatus*) and various non-target (Chironomidae, other macroinvertebrates, protozoa) organisms has focused on the same six wetlands ("monitoring objects")[16,17,27-29]:

Three Bti treated wetlands:

1) Wet meadow with predominately grass vegetation (Nordmyra)
2) Wet meadow with predominately reed and Carex vegetation (Laggarbo)
3) Alder swamp (Valmbäcken)

Three reference (untreated) wetlands

1) Wet meadow with *Myrica gale*, reed and carex vegetation (Lusmyren)
2) Wet meadow dominated by grass (Fågle)
3) Alder swamp (Koversta)

5.2 Summary of treatment and monitoring activities

1) These wetlands were generally monitored from May to September each year, and analyses have been published covering the years 2002-07.

2) The treated wetland wetlands were not subjected to Bti in all years. All three were treated in 2002 and 2006, two of the three were treated in 2003 and 2005, and no treatment was carried out in 2004 and 2007. Treatment dates also varied. For example, two treatments were carried out in 2002, on the 13-14th July, and on the 29-1st August. One treatment was performed on the 12th May in 2006, presumably in response to an early mosquito outbreak.

3) Target species (nuisance mosquitoes) were sampled using two methodologies: (i) 3 light/CO2 traps per locale, trapping monthly for two nights June-August, and (ii) scoop sampling of larvae to track the need for spraying

4) NTO insects with a flying adult stage (predominantly chironomids) were sampled using modified "Mundie's" emergence traps, left on the wetlands during the monitoring period (floating on the water during the aquatic phase, resting on the ground during the terrestrial

phase). These were emptied weekly May to September. Originally 10 such traps were placed out per wetland, but only four were sorted on each sampling date, due to resource limitations. The four sites were chosen to represent a range of environmental conditions.

5) NTO Protozoans were sampled from water samples collected using a 100mL cup, 5 sites per wetland

5.3 Brief evaluation of previous monitoring scheme

(5.3.1) Strengths of the previous monitoring scheme:

(1) The previous monitoring scheme had very good temporal coverage, with weekly sampling during the peak period for mosquito and chironomid emergence.
(2) Additionally, within years there was coverage of both the wet and dry phases of the wetlands, reflecting the fact that a significant proportion of the potentially sensitive NTOs (the terrestrial chironomids) inhabit the drier phase.
(3) Taxonomic resolution was very high, including for the often cryptic Chironomidae.
(4) The scheme used well-established methodologies, based mostly on emergence trap sampling.
(5) The capacity to leave the traps on the wetlands allows for flexibility in sampling dates, and ensures that important peaks in emergence activity wont be missed.

(5.3.2) Limitations of the previous monitoring scheme

(1) The program had very limited spatial coverage. This is true both at the whole-wetland scale, with only 3 treated and 3 reference wetlands, and at the within-wetland scale, with only four emergence traps sorted per site.
(2) This lack of spatial replication limits coverage of the range of variation between and within wetlands, which compounds the lack of statistical power in the design (see Section VI), and carries with it a high risk of making Type II statistical error (which occurs when a null hypothesis is not rejected despite being false – see Section VI).
(3) Efforts to standardize habitat conditions (e.g. with an even sampling of wet and dry conditions, or different wetland vegetation types) also appear to have been limited (and may in practice be difficult).
(4) Apart from measurements of water level, very little supporting data was collected. Such measurements could include water/soil chemistry, detailed vegetation surveys,

water and air temperature, or the effective concentration of Bti at the local scale. The lack of such data limits the possibilities for explaining the large variation in the data.

The emergence trap sampling method used for Insect NTOs, while flexible, also has limitations, particularly with respect to sampling patchily distributed organisms. For example, fixed emergence traps would be unsuitable for sampling mosquitoes, due to the extremely patchy distribution of larval mosquitoes within the flooded wetlands. This patchy distribution relates to the patchy occurrence of microtopographical depressions in the landscape (J. Lundström, Pers. Comm.), which remain aquatic for a time sufficiently long for the mosquitoes to complete their development. Fixed emergence traps would risk missing such microtopographical "hotspots" for larval mosquitoes, and it is notable that adult mosquitoes were instead sampled using combination light and CO2 traps, which attract blood-sucking adults from a wide area.

The distributions of some NTOs, such as the truly aquatic chironomids, may also reflect microtopographical or other microhabitat variation, and thus be very patchy within wetlands. It is thus unclear whether the emergence trap method is suitable for the representative sampling of all important NTOs, particularly with only four replicates per wetland.

5.4 Evaluation of previous statistical analyses

Statistical analyses presented in publications from the monitoring program[16,17,27,28] have included:

(1) Extensive use of Analysis of Variance (ANOVAs), to assess how variation in response variables such as total Chironomid abundances, and abundances of individual subfamilies and species, vary with factors such as year, week, and wetland category (Bti treated vs reference).

(2) The use of Variance Components, primarily in an attempt to quantify the amount of variation in the response variables that can be attributed to Bti treatments

(3) Limited use of multivariate "cluster analysis" for categorizing multivariate community structures in the wetlands.

In all cases, there are several areas where the analytical approaches could be improved upon, to increase the variance explained, the statistical power of the tests, and the range of parameters considered.

(5.4.1) ANOVAS

A well-specified ANOVA model offers great possibilities for partitioning variance and maximizing the chance for detection of significant effects[30]. Unfortunately, the ANOVA models used in publications from the monitoring program are not well described, and no results are presented in detail (*F*-statistics, significance values etc). This makes it difficult to assess exactly what ANOVA model was fitted, though it seems clear that there is substantial scope for refining these analyses:

1) Ideally, analyses of the Bti treatment applied at a specific, well-defined point in time would include an explicit comparison between the response variable before and after the treatment application[31,32]. There is no mention of such a comparison in the existing analyses. Rather it appears that time *per se* has been fitted as a variable (either factor or covariate), with no explicit identification of the before and after treatment periods. Any significant effect of time could thus reflect fluctuation WITHIN the time periods before and after the treatment was applied. This then necessitates some form of post-hoc assessment of whether the time affect was driven by the Bti treatment itself.
2) The sampling scheme implies multiple levels of replication which are partly nested (eg: whole wetlands are the replicates for Bti treatment, with repeated sampling points in time nested within the wetlands), and at least one random blocking factor (the wetlands are effectively blocks of emergence trap subsamples). Blocking and nested factors divide up variation among different spatial units and can help to account for random, "background" variation, and this helps increase the power of tests for fixed factors such as the Bti treatment[30,33]. It is not clear that any of these characteristics of the sampling scheme were accounted for in the ANOVA models.
3) The grouping of wetlands into two factor levels (Bti treated and untreated) obscures variation within and among wetlands in, for example, the actual concentration of Bti on the ground, flood extent, and so on. The incorporation of appropriate covariates relating to these variables (eg: water level, Bti concentration) might further reduce unexplained variance[30,32]. Analysis of Covariance using water level and time as covariates was employed in some publications, but future biomonitoring should aim to collect supporting data that can increase the range of potential covariates.

(5.4.2) Variance Components analysis

Variance Components analyses are typically used to assess the amount of variation associated with random factors in hierarchical, blocked designs[34]. Specifically, variance components analyses can be used to assess whether a random factor explains a significant amount of variation in a model, and thus whether it improves the model's explanatory power. Every factor in a variance components analysis is thus, by definition, random. In previous publications from the Dalälven monitoring program, variance components has been used to assess the variation associated with Bti treatment, which is clearly a fixed factor, imposed in a controlled manner by human agency. The amount of variation explained by a fixed factor is better focused on terms from the ANOVA analysis, such as the relative size of the mean square, or using tests developed specifically for quantifying the variance explained by a fixed factor, such as the partial-Eta of Cohen's F statistics[30,35].

(5.4.3) Multivariate analyses

In general, the data analysis in previous publications shows an over-reliance on univariate approaches for data that is inherently multivariate. Apart from a cluster analysis in one paper, there are no analyses investigating effects of the Bti treatment on community structure, and little consideration of diversity. This risks missing potential effects of the Bti treatment in multivariate space[36]. For example, 10 Univariate ANOVAs conducted separately on the abundances of 10 individual species might find that a given factor is not significant. However, these species do not interact independently of one another, and their responses should accordingly be analyzed together. Multivariate approaches such as MANOVA and ANOSIM (Analysis of Similarities) have potential for detecting changes in community structure (eg: associated with correlated changes in abundances of suites of species) that Univariate analyses miss[36,37]. Ordination techniques also increase the possibilities for investigating relationships between community structure and a suite of additional, environmental covariables[35,36]. Such techniques are particularly useful when community change is gradual along gradients, rather than sudden, driven by some category change.

(6) Statistical analysis of existing data

6.1. Background information

(6.1.1) Aims and Scope

We have carried out statistical analyses of data collected as part of the previous biomonitoring program from 2002-07, to address the following general aims:

4) To apply a Before-After Control-Impact (BACI) design to ANOVA models for analyzing the data
5) To assess the statistical power of those models at the actual levels of replication used in the previous monitoring program, and to analyze the levels of replication required to increase statistical power to reasonable levels
6) To demonstrate some alternative designs and analytical techniques that could be applied to the data.

Note that these aims do NOT include the reworking of existing analyses, to replace those already published. This was beyond our terms of reference for this report, and would require a much deeper familiarity with the data than we could develop within a short time period.

Accordingly: ALL analyses presented here are to be considered *primarily demonstrative in character*, and are *NOT intended for drawing general inferences* on the effects of Bti treatment or any other variable, and should not be disseminated beyond this report.

(6.1.2) Statistical Power & Type II statistical error

The power of a statistical test is a quantification of its capacity for avoiding a so-called Type II statistical error. A Type II statistical error occurs when a statistical test fails to reject a null hypothesis that is, in reality, false (i.e. fails to detect a real difference between two groups of data points)[30,38]. Type II errors are more likely to occur when:

(i) The data is highly variable
(ii) The effect of interest is small, particularly relative to the magnitude of other sources of variation in the data
(iii) Replication is limited

Thus, the real effects of any variable are hardest to detect when sample sizes are small, especially when the data is more variable, and especially when the effect of interest is relatively subtle. This requires consideration when interpreting results from the Dalälven biomonitoring program:

(i) Background spatio-temporal variation among the chironomid populations of The Dalälven is extremely high. For example, total chironomid abundance averaged only 12 individuals per emergence trap in 2002. However, across all emergence traps, total abundances ranged up to 270 individuals.

(ii) While some chironomid species seem vulnerable to Bti at operational doses, most species are not, *a priori,* expected to be strongly affected at operational doses (see Appendix I, this report). This implies that for most species, any variation in abundances caused by Bti is likely to be subtle, especially relative to background variation.

(iii) The initial monitoring program was characterized by limited spatial replication both within (4 emergence traps per wetland) and between (6 wetlands total) wetlands.

Statistical power can be quantified as a percentage.

A test with 95% statistical power will be associated with only a 5% risk of making a Type II statistical error. In contrast, a test with statistical power of 15% carries a substantial risk of failing to detect an effect when an effect really exists, and inferences based on such a weak test should be made with great caution[30,39].

Power analyses allow quantification of the statistical power of any current test to detect a nominated effect size at the observed levels of variability in the data[39]. Additionally, in cases where statistical power is low, power analysis can be used to assess how much additional replication would be needed to raise statistical power to acceptable levels (generally, 90+%).

(6.1.3) Before-After Control-Impact Analysis

Ideally, a study quantifying the effects of a human perturbation applied at a specific point in time should employ a replicated "**multiple Before-After Control-Impact" (mBACI) design**[32]. In other words, a design with sampling before and after the Bti treatment is applied, and in replicated reference and treated wetlands. This allows the separation of variation associated with the Bti application in the treated wetlands, from background variation occurring over the same time period across both impacted and control sites[31,32]. It seems that such a design was not used in analyzing data from the previous monitoring scheme, though the sampling design employed was amenable to such an analysis, at least in some years. Such a design requires the use of a repeated-measures or split plot ANOVA, with replicate sample points in time nested within the appropriate spatial units (in this case, wetlands)[30,32].

(6.1.4). Choice of data analyzed: Focusing on 2002

A major purpose for our power analyses was to give guidelines on the appropriate levels of replication required for future biomonitoring of the effects of Bti treatment. Accordingly, it was decided to focus on those years when all three Bti wetlands were treated. In the dataset we received, there were two such years: 2002 and 2006. However, the 2006 Bti treatment was applied on the 12th of May, and there was only one week of pre-treatment samples, which is not adequate for a meaningful mBACI model. Accordingly, it was decided to focus on 2002, when the Bti treatments were applied in the second half of July, allowing application of an mBACI model with extended periods of data collection pre- and post-treatment.

6.2. ANOVA analyses using an mBACI model

(6.2.1) Description of the model

Univariate ANOVAs were conducted for the following response variables:

1) Total chironomid abundance
2) Total chironomid species richness (number of species per sample)
3) Shannons diversity, an index of diversity which accounts for the relative abundances of species as well as species numbers
4) Abundance of a common terrestrial species *Pseudosmittia angusta*
5) Abundance of the common aquatic predator *Ablabesmyia longistyla*

These variables were chosen to ensure the assessment of statistical power across a range of potentially important univariate indices characterizing different aspects of the chironomid assemblages (diversity and abundance, including of the most common species), and contrasting in how much they varied across replicates. All variables except for the Shannon's diversity index were natural log transformed prior to analysis, in order to satisfy the normality and homoscedasticity assumptions of ANOVA.

These variables were analyzed within an mBACI design, using a split-plot, mixed model analysis of variance. This model is characterized by the use of both fixed and random factors, and by the use of nested variables to divide variance among different spatio-temporal levels of organization (Table 1).

Random factors are those factors whose levels represent a random subsample of all possible levels[30,35]. These are primarily blocking variables that help to define the hierarchical nature of the model. In this model, there is one random factor fitted as a main effect:

1) Wetland, with six levels corresponding to the six wetlands studied. These six wetlands represent a subsample of all possible wetlands that could have been studied, and are the true replicates for the CI treatment.
2) All interactions between wetland and the fixed effects are also random

An additional random variable that could potentially be fitted is a factor denoting pairing among wetlands. Such pairing can help model variation related to geographic proximity or vegetation cover[40]. For example, the reference alder swamp Koversta could be paired with the nearby treated alder swamp Valmbäcken. Presently, with only six wetlands total there is insufficient replication to allow incorporation of pairs in the mBACI design, but a future biomonitoring scheme with more replicate wetlands could include explicit pairing of wetlands.

The fixed factors are those factors whose levels were imposed by human agency:

1) Bti Control-Impact (CI): with two levels, representing the division of the six wetlands into two categories: those treated with Bti, and untreated references
2) Bti Before-After (BA): with two levels, representing the division of samples within wetlands into those taken before application of Bti to the treated wetlands, and those taken after.

Specifically, the *before* period comprises the four weeks prior to the first application of the treatment, and the *after* period comprises the four weeks following application of the first treatment. The length of these periods were chosen based on periods of minimal background change in community composition prior to and after the treatment (which can be assessed based on Bray-Curtis similarity, see section 6.4 below). However, ideally this choice should be based, at least in part, on knowledge of the phenology of the organisms.

Further fixed factors are weeks nested within the BA treatment, comprising four levels in each case, and the interaction of weeks with CI. In this case, the weeks are not a random subsample of all possible weeks, as the choice of which weeks are included is dictated by when the Bti treatment was executed.

The full MBACI design is depicted in table 1, and has the following features:

1) CI is tested against the CI-Wetland interaction, since the Wetlands constitute the true replicates for this factor.
2) BA and the BA-CI interaction are tested against the BI*Wetlands interaction, this ensures that the BA-CI comparison is first constrained within individual wetlands, before the global difference is tested for.

The key terms for making general inferences are the fixed effect tests:

- CI tests whether there is an overall difference between Bti treated and reference wetlands, pooling across all times
- BI tests whether there is an overall difference between the period before and after application of the Bti treatment, pooling across all wetland types
- BA * CI tests whether the responses of the Bti treated and reference wetlands differ when comparing the periods before and after application of Bti to the treated wetlands

Of these, the **BA-CI interaction** is the most important, as it separates out variation associated with the Bti application in the treated wetlands, from background variation occurring over the same time period across both impacted and control sites[32].

Table 1: Split plot design used for univariate multiple Before-After Control-Impact (mBACI) ANOVAs of Bti monitoring data from 2002. Error terms are random effects, shown in italics, against which the preceding fixed terms, shown in plain text, are tested. Nesting is specified using brackets.

Factor	Degrees of Freedom
Between plots stratum: CI x Wetland Stratum	
Wetland treatment: Bti Control-Impact (CI) comparison	1
ERROR: Wetland(CI)	*4*
Within plots stratum: Before-After periods nested within wetlands	
Before-After (BA) Bti Treatment comparison	1
BA * CI interaction	1
*ERROR: BA * Wetland(Treatment)*	4
Times within plots stratum: weeks nested within before and after periods	
Week(BA)	6
Week(BA) * CI	6
*Week(BA)*Wetland(Treatment)*	24

A feature of this design is that variation is partitioned according to different levels of spatio-temporal variation. With only 6 wetlands total, the statistical tests for most fixed effects are not well-replicated, though still adequate to detect some stronger effects (Section 6.3, below).

Note that in this model, the individual spatial sampling points within wetlands (comprising 4 emergence traps per wetland) do not contribute to the power of any statistical test. Their main role is rather to provide coverage of the range of conditions within wetlands, and thereby improve the precision of mean estimates[30,41]. As they do not contribute to replication, there is substantial capacity for saving sorting effort by pooling and subsampling emergence traps within wetlands (see Section 8 below).

Two types of ANOVA were conducted:

(1) ANOVA of the **full data set**
(2) ANOVA of the **terrestrial** (non-inundated) replicates only.

The second ANOVA was conducted by excluding all emergence trap replicates from the aquatic phase of the wetlands (ie: when water level measurements >0). This allows assessment of how a narrower focus on a single ecosystem phase alters variability in the data, and thereby alters significance values and statistical power. The terrestrial ANOVAs were conducted for the composite variables only (Abundance, Richness, Shannon's Diversity), as data distributions became non-normal for the individual species with exclusion of some replicates, even after log-transformation.

All ANOVAs were conducted using the General Linear Model syntax option in SPSS 19.0.1 for Macintosh.

(6.2.2) ANOVA results

Due to the low statistical power of the tests, the ANOVA tables below highlight all significance values >0.1, to emphasize values at or near the 0.05 significance levels. Significance levels between 0.05 and 0.1 represent cases where substantial systematic variation is associated with a factor, but where further replication is needed to definitively confirm or reject the null hypothesis[30,35].

Table 2 FULL DATA SET: Significance test results from split-plot MBACI ANOVA analyses of chironomid emergence trap data from 2002. Probability values are given for p<0.1, to highlight cases both at and near significance at ∝ = 0.05. Random effect terms are shown in italics, and were used as error terms for the preceding fixed effects, shown in non-italics. Species *P. ang.* = *P. angusta, A. long.* = *A. longistyla.* Other abbreviations: CI = Control-Impact, BA = Before-After, df = degrees of freedom, Abund. = abundance, ns = non-significant (p >>0.1).

	df	ANOVA significance tests				
		Abund.	Richness	Shannon	*P. ang.*	*A. Long.*
Between Wetlands Stratum						
CI	1	ns	ns	ns	ns	ns
Wetlands(CI)	*5*	*ns*	*ns*	*ns*	*ns*	*0.001*
Within wetlands Stratum						
BA	1	ns	ns	ns	0.051	ns
BA * CI	1	ns	ns	ns	ns	ns
*BA*Wetlands(CI)*	*4*	*ns*	*ns*	*ns*	*0.09*	*ns*
Time within wetlands stratum						
Week(BA)	6	ns	ns	ns	ns	ns
Week(BA)*CI	6	ns	ns	ns	ns	ns
*Week(BA)*Wetland(CI)*	*24*	*ns*	*ns*	*ns*	*0.057*	*ns*

Significance levels from analyses of the full data set are presented in Table 2:

1) No fixed or random effect was significant for the composite variables (Total abundance, Richness, Shannons diversity).
2) Some random effects were at or near significance for the two individual species tested: *P. angusta* and *A. longistyla,* reflecting background variation among wetlands and sample dates
3) The fixed effect BA was near significance for *P. angusta*. This reflected a substantially higher abundance across all wetlands before (9.7±4) compared with after (0.1±0.05) the Bti treatment. Since there was no interaction with the CI treatment, this difference is most likely to represent a background phonological decline in this species, and not a treatment effect of Bti.

Significance levels from ANOVA analyses of terrestrial emergence trap data are presented in table 3, for the three composite variables:

1) Additional random and nested factors have neared significance, reflecting variation among wetlands, and among sampling dates within BA periods
2) The fixed effect BA * CI has neared significance for abundance.

Table 3 TERRESTRIAL DATA ONLY: Significance test results from split-plot MBACI ANOVA analyses of chironomid emergence trap data from 2002, with immersed (aquatic) replicates excluded. Probability values are given for p<0.1, to highlight cases both at and near significance at ∝ = 0.05. Random effect terms are shown in italics, and were used as error terms for the preceding fixed effects, shown in non-italics. Abbreviations as for Table 1.

	df	ANOVA significance tests		
		Abundance	Richness	Shannon
Between Wetlands Stratum				
CI	1	Ns	ns	ns
Wetlands(CI)	*5*	*0.09*	*ns*	*ns*
Within wetlands Stratum				
BA	1	Ns	ns	ns
BA * CI	1	0.07	ns	ns
*BA*Wetlands(CI)*	*4*	*Ns*	*ns*	*ns*
Time within wetlands stratum				
Week(BA)	6	Ns	ns	ns
Week(BA)*CI	6	Ns	0.091	ns
*Week(BA)*Wetland(CI)*	*24*	*Ns*	*ns*	*Ns*

The significant BA-CI interaction indicates that the responses of the terrestrial assemblages differed between treatment and control wetlands before and after application of Bti to the treated wetlands. Specifically, whereas abundances appeared to decline in control wetlands in the after period, they increased in the treated wetlands. The capacity of the terrestrial-only tests to detect additional effects reflects an increased statistical power (see Section 6.3, below)

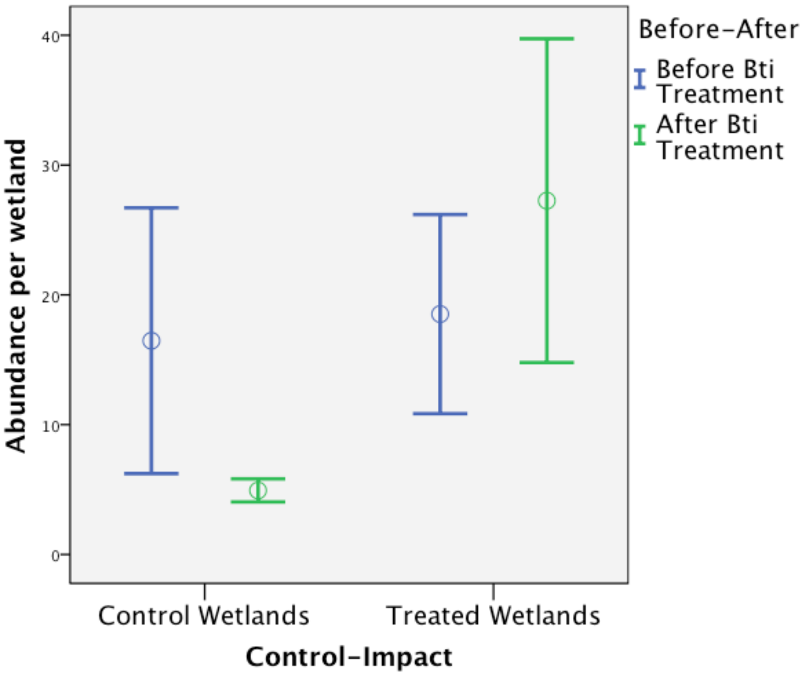

Figure 1 Abundance (mean ± SE) of terrestrial-phase chironomids in control and treated wetlands, before and after the application of Bti.

6.3. Power analyses of the ANOVA models

(6.3.1) Description

Power analysis require 3 pieces of information[39]:

1. The variation (standard deviation) associated with measurement of a factor (e.g. the variation in chironomid abundances from control and treated wetlands)
2. The sample size (replicate number) used in estimating that variation (e.g. the number of whole wetland replicates for the reference and treated categories)
3. The size of the effect which needs to be detected (e.g. the size of the change in chironomid abundances that an ANOVA model SHOULD be able to detect)
4. The significance level ($p = 0.05$)

From this information, the power analysis can estimate:

1. The observed power of a statistical test from an ANOVA, given its sample size and the levels of variation in the data
2. In cases where the observed power is low, how much additional replication would be required to raise statistical power to acceptable levels

The power analyses presented here focus on one term from the previous ANOVA models: the BA-CI interaction. This is the key interaction for assessing whether Bti treatments affects chironomid communities[32], but is not well replicated in the current model (with only 4 residual degrees of freedom at the within plots level).

For each power analysis, two effect size scenarios are presented for each response variable:

1. An effect size of 10%, entailing a 10% change (either positive or negative) in the response variable
2. An effect size of 66%, entailing a 66% change (either positive or negative) in the response variable

The larger effect size (66% change) approximates the effects observed in some previous studies of Bti treatments on ecological parameters. For example, chironomid richness declined by approximately 60% following the application of Bti in Minnesota wetlands, and birds consumed 66% fewer nematoceran dipeterans in French wetlands treated with Bti[18]. This is also regarded as an ecological meaningful effect size, given that 66% reductions in quantities of nematoceran Dipterans in bird diets were associated with reduced breeding success. A 10% effect size would represent a more subtle effect size of uncertain ecological importance. However, even if a 10% effect size is of minor short-term ecological importance, it is still of great value for a biomonitoring scheme to detect such changes, which can act as early warning signals for systems subjected to repeated Bti treatments.

The following information is presented from the power analyses:

1. Observed power at the actual level of replication used in the previous biomonitoring scheme (n = 6 whole wetlands)
2. The additional replication required, if any, to raise power to 50% (ie, to reduce the chance of a Type II error to 50%)
3. The additional replication required, if any, to raise power to 95% (ie, to reduce the chance of a Type II error to only 5%).

Power analyses are presented here for the BA-CI interaction, for all ANOVA models presented in the previous section, including comparison of the full with the reduced data set. The BA-CI interaction is the single most important factor for assessing whether Bti has had an impact on NTO assemblages[32]. In all the charts presented below, the x-axis plots replication at the wetland scale, with a guideline plotted at replication n = 16, representing a potential maximum practical level of wetland level replication at Dalälven.

The power analyses were conducted using the "Sample Size and Power" algorithm in JMP 8.0.1 for Macintosh.

(6.3.2) Total abundance

Relationships between replication and statistical power for total abundance are presented in Figure 2:

- For tests attempting to detect a 66% change in abundance in the total data set, statistical power was high (at 95%) even for the existing sampling scheme (ie, where sample size n = 6) (Fig. 2A)
- However, for tests attempting to detect a 10% change in abundance, statistical power was low, at only 35% (Fig. 2A)
- To increase the power to detect smaller changes (Fig. 2A), it would be necessary to increase the number of wetlands sampled to between 8 (to reduce the chance of a Type II error to 50%) and 20 (for a Type II error rate of 5%)
- For the analyses focused on the terrestrial data sets only (Fig. 2B), statistical power was high for detecting both small (60% power) and larger (95% power) effect sizes. To improve the capacity to detect smaller effect sizes, it would require an increase in whole wetland replication to 14.

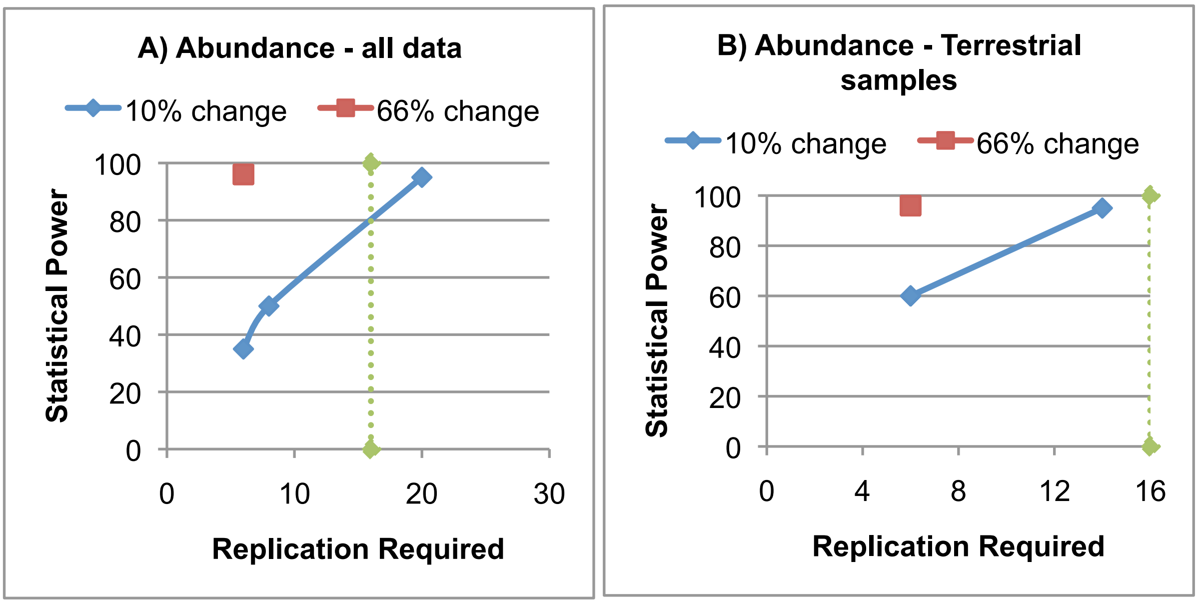

Fig 2: Relationship between replication and statistical power of the BA-CI interaction for total abundance data from ANOVAS including (A) all emergence trap data and (B) data from terrestrial emergence traps only. The red and the blue lines plot data for two different effect-size scenarios. The green dotted line corresponds to the n=16 level of whole wetland replication, a possible practical maximum at Dalälven.

(6.3.3) Species Richness

Relationships between replication and statistical power for species richness are presented in Figure 3:

- Tests attempting to detect a 66% change in richness in the total data set had high statistical power even for the existing sampling scheme (ie, where wetland replication n = 6) (Fig. 3A). However, power was very low for tests attempting to detect a 10% change, at only 13% (Fig. 3A)
- To increase the power to detect smaller changes (Fig. 3A), it would be necessary to increase the number of wetlands sampled to somewhere between 26 (for a Type II error to 50%) and 76 (for a Type II error rate of 5%)
- For the analyses focused on the terrestrial data sets only (Fig. 3B), statistical power was high (95% power) for detecting the larger effect size. However, for the smaller effect size the statistical power was only 26%. To improve the capacity to detect smaller effect sizes, it would be necessary to increase the number of wetlands sampled to between 10 (for a Type II error to 50%) and 34 (for a Type II error rate of 5%).

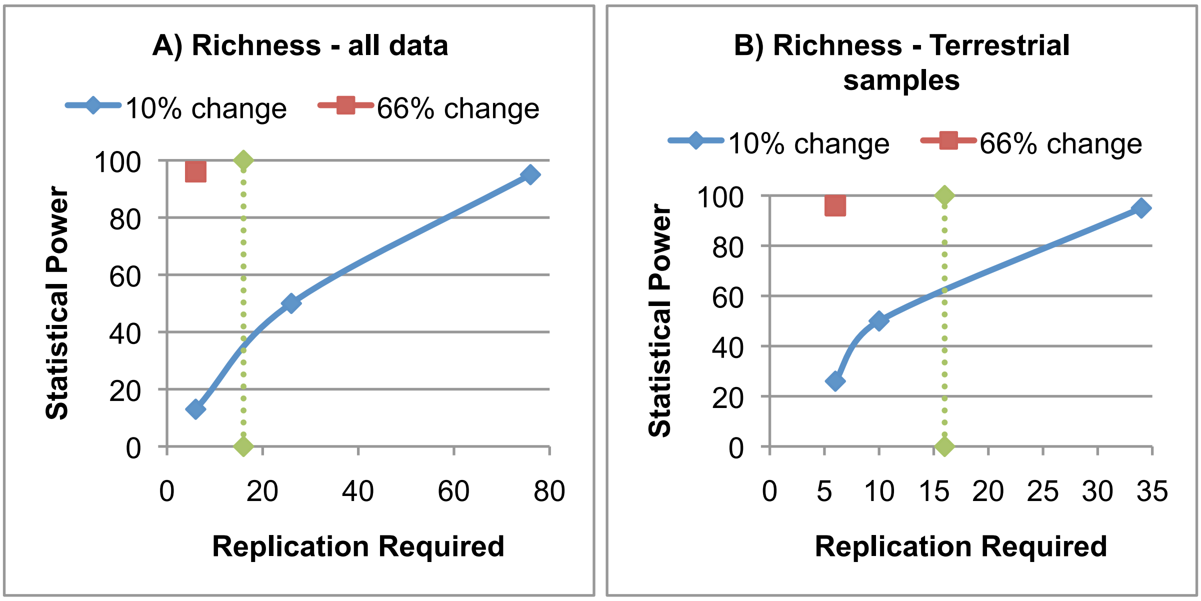

Fig 3: Relationship between replication and statistical power of the BA-CI interaction for total species richness data from ANOVAS including (A) all emergence trap data and (B) data from terrestrial emergence traps only. Line and symbol coding as in Fig. 2.

(6.3.4) Shannon diversity

Relationships between replication and statistical power for Shannon diversity are presented in Figure 4. Shannon diversity was highly variable, reflecting substantial fluctuations in species relative abundances among wetlands and sample dates. Accordingly, statistical power was uniformly low.

- Tests attempting to detect a 66% change in Shannon diversity in the total data set had low statistical power (16%) for the existing sampling scheme (ie, where wetland replication n = 6), and very low power (<1%) for tests attempting to detect a 10% change (Fig. 4A)
- To increase the power to detect smaller changes (Fig. 4A), it would be necessary to increase the number of wetlands to between 502 (for a Type II error to 50%) and 1652 (for a Type II error rate of 5%)
- For the analyses focused on the terrestrial data sets only (Fig. 4B), statistical power was low for detecting both larger (31% power) effect sizes, and smaller (7%) effect sizes To improve the capacity to detect smaller effect sizes, it would be necessary to increase the number of wetlands to between 202 (for a Type II error to 50%) and 760 (for a Type II error rate of 5%)

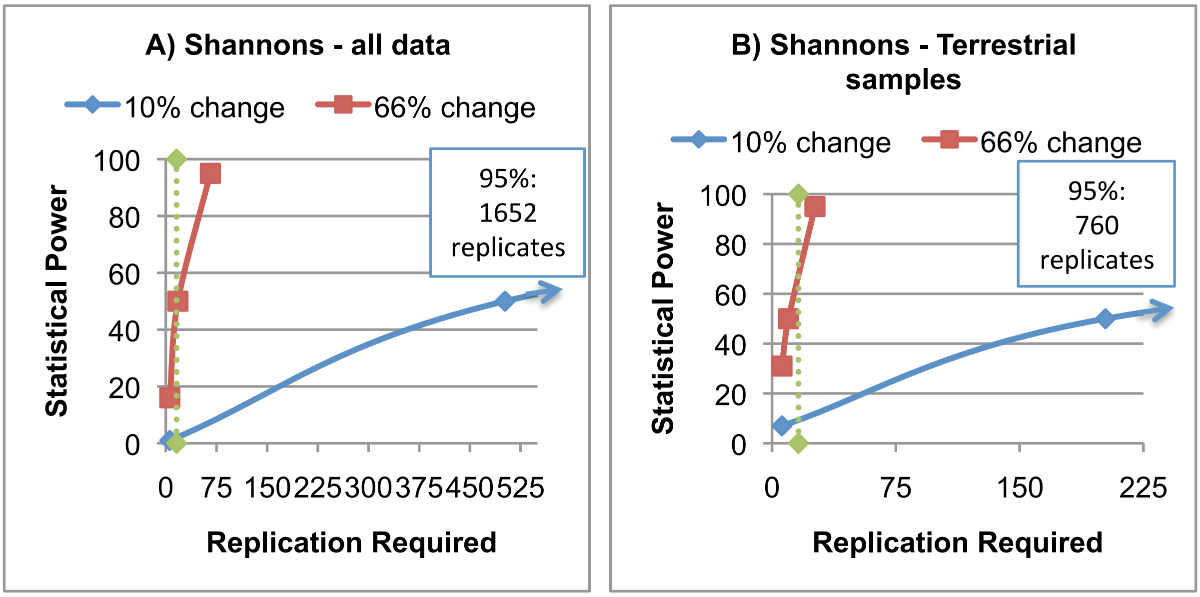

Fig 4: Relationship between replication and statistical power of the BA-CI interaction Shannon's diversity index data from ANOVAS including (A) all emergence trap data and (B) data from terrestrial emergence traps only. Line and symbol coding as in Fig. 2.

(6.3.5) Abundance of Pseudosmittia angusta and Ablabesmyia longistyla

Relationships between replication and statistical power for ANOVAS on abundances of two common species, *P. angusta* and *A. longistyla*, are presented in Fig. 5

- Tests attempting to detect a 66% change in the abundance of *P. angusta* in the total data set had low statistical power (45%) for the existing sampling scheme (ie, where wetland replication n = 6), but the increase in replication required to improve statistical power to 95% is relatively modest (n = 14). However, the power to detect a 10% change was very low (14%), and the increase in replication required to increase power to 95% (for a Type II error rate of 5%) is substantial (n=100) (Fig. 5A)
- Statistical power for tests detecting any change in *A. longistyla* abundances was extremely low for the existing sampling scheme (<1%), and would require enormous increases in whole-wetland replication to improve to even moderately acceptable levels (Fig. 5B).

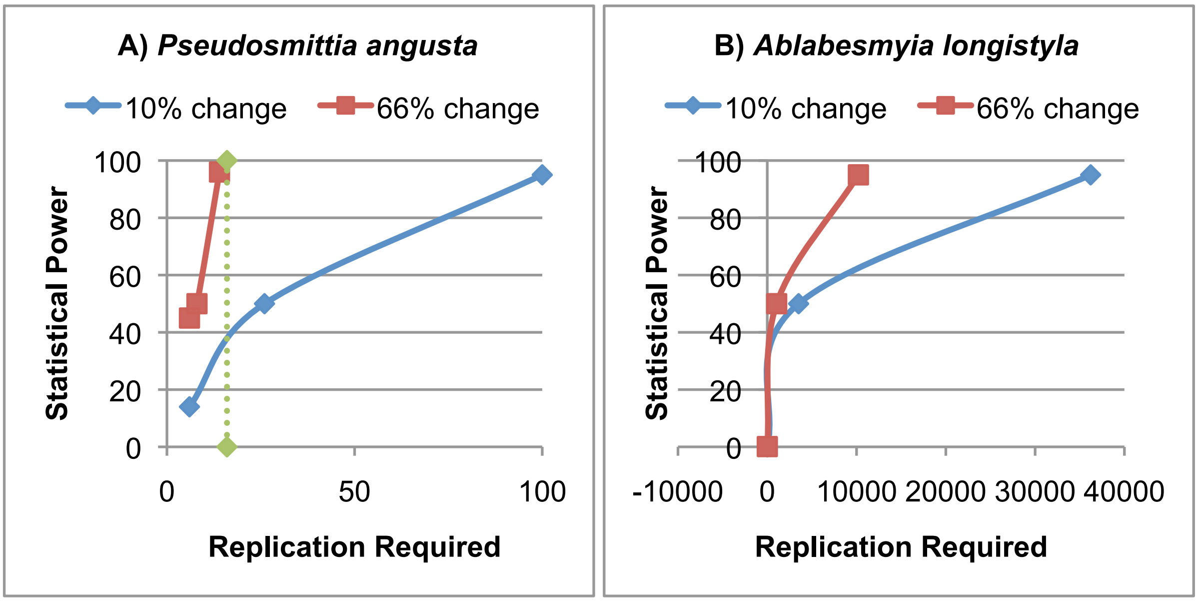

Fig 5: Relationship between replication and statistical power of the BA-CI interaction for (A) *Pseudosmittia angusta* and (B) *Ablabesmyia longistyla* data. Line and symbol coding as in Fig. 2.

(6.3.6) Power analyses: Summary

1) The power to detect larger effects sizes in the BA-CI interaction was high for some parameters (e.g. abundance and richness), even in the existing sampling scheme with only 6 whole wetland replicates.
2) However, the power to detect more subtle changes was generally low (<45%).
3) By narrowing the focus of the analyses to the terrestrial wetlands only, statistical power uniformly improved. This reflects the reduction in variation associated with focusing only on the terrestrial assemblage, compared with the full data set which combines the terrestrial and aquatic assemblages
4) By separating analyses of the terrestrial and aquatic phases of the wetland, it would be possible to attain reasonable or good statistical power for several variables with only a modest increase in total wetland replication (maximum n=16)
5) The data for *A. longistyla* highlights the risks of conducting analyses for individual species, even when their total abundance is high

Implications of these analyses for interpreting the ANOVA results (Section 6.2, above), include:

1) Results for the whole data set (Table 2) carry a low risk of Type II error (<5%) for some variables (abundance and richness), if the aim is only to assess strong effects of Bti. Thus, it could be safely concluded that Bti has had no marked effects (±66%) on chironomid abundance or richness.
2) Results from the whole data set carry a high risk of Type II error for all variables, when the aim is to detect more subtle Bti effects. For example, the risk of a Type II error (failure to detect a change when a change in fact occurred) in detecting a 10% change in the abundance data is 65%.
3) For some variables (Shannon's diversity, *A. longistyla* abundance), the existing scheme had very little statistical power, and prospects for increasing power by increasing whole-wetland replication are unrealistic
4) Results for the analysis focusing on terrestrial data set only illustrate the substantial improvements in statistical that could come from separating aquatic and terrestrial monitoring.

6.4 Other analyses: Demonstration of Multivariate Analysis Techniques

The power analysis results for *A. longistyla* (Fig. 5B) illustrate the difficulties involved in analyzing abundances of individual species, even when they are very common. For assessing fluctuations in species abundances, it is often preferable to employ multivariate techniques that analyze the entire assemblage, or large portions of it, simultaneously. Two groups of analyses are presented here: Multivariate Analysis of Variance (MANOVA) and ANOSIM.

(6.4.1) MANOVA

MANOVA is used to test hypotheses are framed in terms of changes in multivariate space[30]:

(1) How do the multivariate abundances of species vary between Bti-treated and reference wetlands?

(2) How do the multivariate abundances of species vary before and after application of the Bti treatment?

A MANOVA analysis is presented here for illustrative purposes. The total number of species that can be fitted to a MANOVA model is constrained by total replication. Thus, this technique is not the most appropriate analysis for the current data set, where the total replication of wetlands is 6, but could become useful for future biomonitoring as total wetland replication is increased.

In order to account for the replication of Before-After periods within whole wetland Control-Impact replicates, we used a split-plot MANOVA, often termed a "doubly multivariate repeated measures ANOVA"[42]. Our Analysis included the 8 most abundant and widespread species, which left very little power for the Error terms (Table 4). Note that like the ANOVA, this MANOVA includes an explicit BA-CI interaction term, which allows separation of the effect of Bti in treated wetlands from background changes occurring across both treated and reference wetlands. The MANOVA was conducted using SPSS 19.0.1 for Macintosh.

Table 4: Output from a split-plot, Multivariate Analysis of Variance of changes in the abundances of the 8 most common species

Factor	Degrees of Freedom	Significance
Between Subjects		
Control-Impact (CI)	4	ns
Error	*1*	
Within Subjects		
Before-After (BA)	4	ns
BA*CI interaction	4	Ns
Error	1	

(6.4.3) MDS ordination, ANOSIM and SIMPER

The second set of multivariate techniques are useful for investigating variation in community structure associated with environmental changes. The techniques of Multi-Dimensional Scaling (MDS) ordination, Analysis of Similarities (ANOSIM) and Similarity-Percentages (SIMPER) all work by first transforming a species abundance matrix using an index of community similarity, typically the Bray-Curtis similarity index[37,43]. This index can then be used to quantify the degree of divergence in community structure between replicates.

Bray-Curtis similarities can be used in MDS ordination to provide a visual picture of the similarity among replicates (Fig. 6). Prior to ordination, the data were pooled into whole wetland BA-CI replicates. Replicates that are closer to each other in the ordination space are more similar in community structure. The ordination of the 2002 data (Fig. 6) indicates a shift in community structure between the replicates sampled before the application of the Bti treatment, situated mostly to the left of the ordination space, and those sampled after, predominantly situated to the left of the ordination space. This shift appeared to affect both untreated and treated replicates alike.

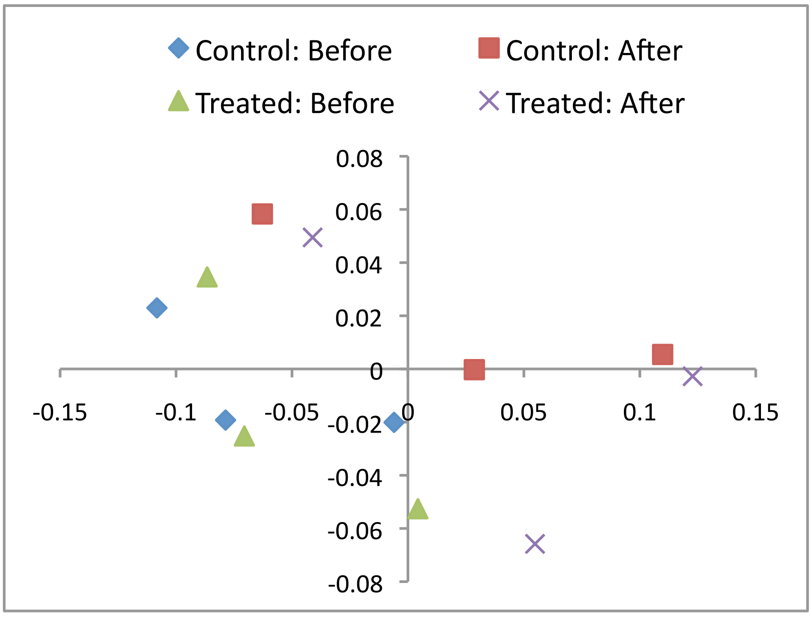

Fig. 6: nMDS ordination of community composition data, with BA-CI categories superimposed using different symbols. Replicates placed closer to one another are more similar in community structure.

Following ordination, divergence in faunal composition among BA-CI replicates were tested statistically using ANOSIM. ANOSIM tests for differences between similarity matrices (generated using Bray-Curtis similarities), through calculation of the global test-statistic "Rho". Rho varies between 0 and 1, with values greater than 0.15 generally not arising by chance[43]. A Rho value closer to 1 indicates that similarities are greater within than between sets of replicates, while a value of zero indicates uniform similarities between and within sets. The ANOSIM analyses confirm the ordination results, with assemblages differing between the before and after periods (Rho = 0.16, $p < 0.001$), but not with treatments (Rho = 0.04, $p = 0.092$).

Finally, the SIMPER (similarity percentages – species composition) procedure can be used to investigate the contribution of each species to mean Bray-Curtis dissimilarity between sites. An example output is presented in Table 5, which lists the species explaining 75% of the disimmilarity between control and treated sites.

Table 5: SIMPER analysis of species explaining 75% of the dissimilarity between reference and Bti treated sites. The contributions of each species to dissimilarity is tabulated, along with the mean abundances of each species in the reference and treated sites

Taxon	Contribution	Cumulative %	Mean abund reference	Mean abund treated
Limnophyes minimus	11.96	14.08	0.411	0.869
Pseudosmittia angusta	9.441	25.19	0.473	0.525
Limnophyes asquamatus	8.235	34.89	0.452	0.531
Telmatopelopia nemorum	6.56	42.61	0.418	0.29
Polypedilum trigonus	6.349	50.08	0.279	0.378
Xenopelopia nigricans	4.488	55.37	0.387	0.103
Paramerina cingulata	3.995	60.07	0.292	0.473
Limnophyes difficilis	3.89	64.65	0.138	0.251
Tanytarsus curticornis	3.267	68.49	0.042	0.707
Tavastia yggdrasilia	3.11	72.16	0.063	0.173
Ablabesmyia longistyla	2.954	75.63	0.042	0.58

(7) GIS analysis of potential reference sites

Future biomonitoring of the effects of Bti in Dalälven requires the careful selection of appropriate reference sites. Ideally, these reference sites would be on another, untreated catchment, but this is not possible in the case of the Dalälven, as there are no comparable catchments having such a well-developed delta within Sweden. Thus, reference sites need to be selected from within the Dalälven catchment itself. This involves multiple challenges, including the changing extent of Bti treatment in the Dalälven region (so that sites acting as references in one year may be targeted for treatment in another), and the high environmental heterogeneity among wetlands.

Geographic Information Systems analysis was used to identify potential reference sites within the Dalälven catchment, but outside of the areas treated by Bti during 2010. The goal was to identify "wetland" localities with characterisitics similar to those that were treated in 2010. Specifically, the GIS analysis identified potential reference sites based on the match between their sensitivity to flooding (annual frequency and extent) in comparison with the current treated wetlands. Up to 20 potential sites were identified from the analysis (Appendix 3, and http://infogis.ma.slu.se/map/mygg/), but during field reconnaissance of these sites, around 20% turned out to be characterized by conifer forest cover (i.e. not true wetlands), and so unsuitable as reference sites, but the remaining 80% appeared suitable.

The location of the potential additional reference areas is mapped at a coarse scale in Appendix 3. The full GIS database, including more detailed information on the location of these sites, is available on request from DASA.

(8) Development of the biomonitoring program

8.1 The problem of Variability

The development of a biomonitoring program for the use of Bti in Dalälven is complicated by the extreme variability characterizing its floodplain wetland systems. Inter-annual variability in the extant and duration of flooding and in temperature characteristics, along with spatial variation among and between wetlands in microtopography and vegetation characteristics, all have strong influences on the development and distribution of populations of both target (mosquitoes) and NTOs[1,17].

This variation poses substantial challenges in planning Bti treatments. The timing, dosages and spatial coverage required for effective mosquito control depend on the total water extent, and on the densities and distributions of the target organisms. Bti treatment in the lower Dalälven is carried out in consultation with a network of local inhabitants who provide up-to-date information on the development of pestiferous mosquitoes, facilitating the optimization of Bti treatment according to the fluctuating need among and within years (Jan Lundström, pers. Comm). Nevertheless, the risk of misdosing cannot be entirely ruled out. Underdosing (application of too little Bti for the volume of water, and/or at the wrong time of the mosquito life cycle) reduces the effectiveness of mosquito control. Overdosing (accidental application of too much Bti for a given volume of water) has potential to raise Bti concentrations to levels with documented effects on NTOs.

This variation also poses substantial challenges for planning biomonitoring, as an appropriate time for commencing before-treatment measurements will be dictated by the application-data of Bti, which can vary among years according to the timing of flooding and the mosquito development rates. Thus, pre-treatment measurements may be required in May during a year of early floods, but in June in a year of later floods[28,29]. Similarly, a suitable locale for sampling aquatic NTOs one year may be unsuitable the following. At Dalälven in particular, the NTO (chironomid) assemblage includes species ranging from fully aquatic to terrestrial taxa[16]. These may be expected to respond differently to the extent of flooding, and to the application of Bti. Terrestrial chironomids live in water logged soils or associated with moist vegetation[13], and a priori might be expected to feed less on Bti delivered in a form designed for consumption in the aquatic environment.

8.2 Guiding Principles in the development of a biomonitoring scheme

A suggestion for the initiation of a new biomonitoring program was delivered to the Swedish Environmental Protection Agency on the 27th of November 2010, and is attached to this report as Appendix 4 (in Swedish). A MODIFIED version of this scheme is given in the Swedish and English "expanded summaries" which open this report. The principles underlying the development of this scheme were based on results from the literature review and survey of international agencies, and on the evaluation of the existing biomonitoring scheme and statistical analyses of existing data. Guiding principles include:

1) The Chironomidae constitute the main NTO group with the greatest potential vulnerability to Bti, and should continue as the focal group for biomonitoring
2) The previous biomonitoring scheme was characterized by extensive temporal but limited spatial coverage. Consequently, statistical power for detecting subtle, and even more marked, effects of Bti on various attributes of chironomid assemblages was limited. Thus, the level of spatial replication should be expanded.
3) Most important is an increase in the number of wetlands sampled, as these constitute the true replicates for the Bti treatment. The power analysis indicates that for some parameters, substantial gains in statistical power can be gained with a relatively modest increase in the number of whole-wetland replicates, from the current six to somewhere between 10 and 16.
4) The use of emergence traps for sampling NTO organisms has been shown to work for these systems, and we suggest that use of this method continue. However, the patchy distribution of NTO across the wetlands necessitates an increase in the number of traps deployed, to increase the chances of sample NTO microhabitat "hotspots". This is needed to improve the precision (reduced variability) in estimation of emergence from individual wetlands
5) More generally, it is notable that previous studies which have detected effects of Bti on NTO organisms have used benthic sampling of NTO larvae, rather than emergence trapping of adults, and we suggest that the feasibility of employing an alternative benthic sampling method be investigated for Dalälven also.
6) Data from the previous biomonitoring program is characterized by much unexplained variation. We suggest the gathering of more supporting data, especially on the effective Bti dosage in different wetlands (if practical), but also

on temperature, water level, and soil and water nutrient status. Such variables could be fitted as covariates in ANOVA analyses, or used in multivariate ordination analyses, to assist in separating out different sources of variation in chironomid assemblages.

7) The power analysis highlighted the value of designing monitoring schemes that explicitly model different sources of variation in the data. Multiple options exist for addressing this, including:
    a. Explicitly separating sampling of the aquatic and terrestrial phases of the wetlands
    b. Explicitly separating sampling of the two main vegetation types in the affected wetlands (alder and wet meadow)
    c. The use of paired reference and impacted sites, to account for geographic proximity, and similarities in vegetation cover and other factors

8) The best practice MBACI statistical models do not directly utilize the individual spatial sampling points within wetlands (comprising replicate emergence traps in each wetland). As they do not contribute to replication, there is substantial capacity for saving sorting and identification effort by pooling and subsampling emergence traps within wetlands[44]. We recommend the use of a "fixed fraction" sampling method (e.g. a "Folsom sample splitter"[45]) that allows inferences on both the diversity and total abundances of chironomids.

9) Even if the direct toxic effects of Bti on NTO are minimal, there are real possibilities that the greatly reduced biomass of mosquitoes delivered to terrestrial environments could have knock-on consequences for other ecosystem properties, particularly on the feeding activity, growth and reproduction of intermediate (invertebrate) and top consumers. The biomonitoring program instituted by the French agency *La Tour du Valet* highlights the multitude of relevant parameters that could be quantified (eg, algal production, densities of intermediate consumers, bird foraging rates etc). We recommend that research be conducted into the value and feasibility of incorporating such measures into a future biomonitoring program at Dalälven.

10) Analyses of data from the biomonitoring program should use a full range of univariate and multivariate statistical approaches, to investigate changes in not only composite metrics, such as total abundance and diversity of target and NTOs, but also in the multivariate community structure of NTOs.

8.3: Description of the Biomonitoring Scheme

The suggested biomonitoring scheme (Appendix 4) includes guidelines for two alternative monitoring programs: a base program and an expanded program. Both programs involve an expansion in the numbers of wetland replicates, the explicit separation of sampling of the aquatic and terrestrial phases of the wetlands, and the use of pooling and subsampling of replicates within wetlands to save sorting and identification-related labour costs.

The *base program* would be carried out only in flooded wetlands, and only in wetlands of the "wet meadow" vegetation type. Originally it was suggested that the base program should include at least 5 each of reference and treated wetlands**, but following our power analyses, we now suggest increasing this number to at least 6, for 12 wetlands total**, and preferably more**.** Other guidelines for the base program include:

- *Non target organisms* would be sampled using 10 emergence traps per site, sampling inundated habitats only (>1cm surface water). Each trap should be deployed for 48 hours and then removed. The traps are randomly redeployed weekly during the most dynamic period (6-8 weeks peak inundation), otherwise biweekly, initially covering the inundation floodplain, then with a focus on remaining water pools as the water levels recede.
- *Target orgnisms* would be sampled using ten combination CO_2-light traps per wetland, deployed randomly over the wetland for 48 hours and then removed, with random redeployment weekly during the active treatment period, otherwise biweekly.
- *Supporting Data* – Physico-chemical data: temperature loggers (3-5 per wetland), water nutrients (5 samples per wetland) and water depth (adjacent to each emergence trap) should be sampled from every wetland at every emergence trap clearance date. Additionally, we now suggest that during periods of Bti treatment, water samples quantifying the effective dosages of Bti also be collected, if possible

A "pooling and subsampling" protocol will be used in all sample processing, whereby within-weland replicates are pooled, and then a set-number of subsamples are selected for further processing and identification[44,46]. In the original proposal suggestion, we recommended the use of a "fixed-count" method. However, since a potential negative impact of Bti could be on total chironomid production, **we now instead recommend a combination "fixed fraction-fixed count" sampling method that allows inferences on both the diversity and total abundances of chironomids (Figure 5).**

The expanded program would i) Add additional wetlands characterized by alder swamp vegetation (3-5 treated and reference, depending on availability), (ii) Add additional emergence traps sampling chironomids of the terrestrial phase (surface water <1cm), (iii) add benthic core sampling, with pooling and subsampling of replicates during processing.

In all cases, we recommend the use of both univariate and multivariate statistical approaches, with care taken to use statistical models that are replicated at the appropriate spatio-temporal scales, and account for multiple sources of variation in the data.

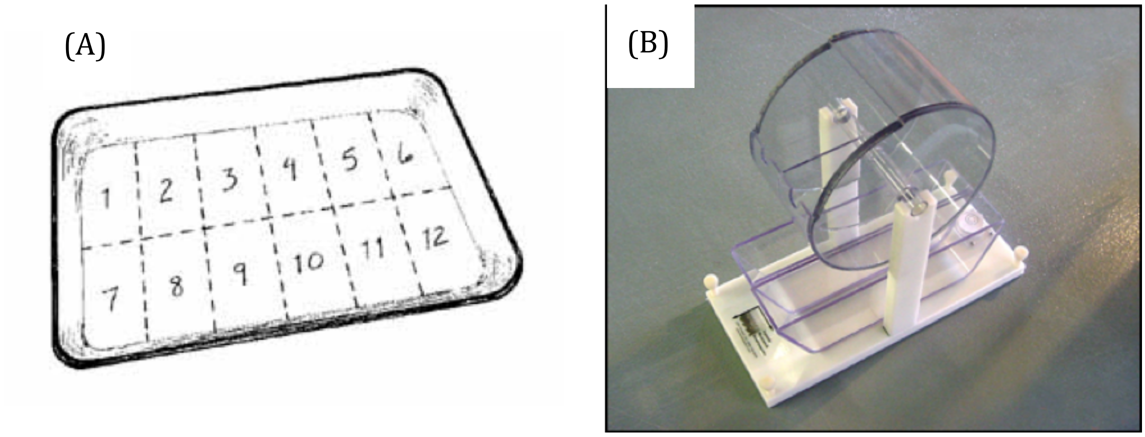

Figure 5A-B: Equipment necessary for two possible “fixed fraction-fixed count” sampling methods.

(A) A sorting tray divided into grids. Subsamples are taken successively from randomly-selected grid-squares, until a predefined “target” number of individuals have been picked out (usually between 300-700). All individuals from the last selected square are picked and identified, even if the final number of animals passes the target number).
Picutre: http://water.epa.gov

(B) A “Folsom sample splitter”, which is used to halve samples into smaller subsamples. Rotation of the cylindrical drum evenly spreads the sample, which is then divided into two by being poured into the two troughs. Once again, successive subsamples are taken until a predefined “target” number of individuals has been picked out.
Picture: www.aquaticresearch.com

(9) Final remarks

The biomonitoring scheme suggested here is preliminary, and ultimately its utility can only be assessed through its execution in the field. For example, practical difficulties in moving around flooded wetlands may render the extent of replication within wetlands proposed here impractical, and sampling schemes and protocols are likely to need refinement. Thus, the development of a biomonitoring scheme for the effects of Bti on ecosystems of the Dalälven requires a long-term commitment, as it goes through an extended period of testing and modification.

A major issue in the development of a future biomonitoring scheme will be the identification and maintenance of reference sites. Given ongoing pressure from local communities to expand the use of Bti in the future, there is a risk that sites chosen as references in the present may be targeted for treatment in the future. While such eventualities may be necessary on humanitarian grounds, the loss of reference sites would also threaten the longer-term integrity of a biomonitoring program. Accordingly, the location of multiple appropriate references for all wetland types (specifically, both wet meadows and alder swamps) that are unlikely to be targeted for future Bti treatment should be a priority.

Development of this biomonitoring scheme has been hindered by a lack of basic information on, for example, the relative vulnerability of terrestrial and aquatic NTOs to Bti, factors governing the microdistribution of NTOs, and the extent of variation in effective Bti dosages on the ground, particularly among different wetland types. The relative utility of different sampling methods (emergence traps versus benthic samples) is also poorly known. Furthermore, the fate of decomposing mosquito corpses in the wetland environment is unclear, as is the importance of both target and NTOs for terrestrial and aquatic foodwebs. For example, it is unclear at present whether a basic biomonitoring scheme should include monitoring of insectivorous birds, potentially vulnerable to a decline in the biomass of emerging aquatic insects. Finally, concerns about the development of resistance to Bti among target organisms[22], and about the persistence of Bti in treated soils and vegetation[21,47], have been expressed in the literature (see Appendix I), and these possibilities also need consideration in implementing any longer-term mosquito control program involving Bti. Thus, apart from the implementation of a basic biomonitoring scheme in Dalälven, there is a substantial need for more research on these and other topics.

(10) References

- **NB: Also see Appendix 1 for full coverage of references relating to Bti and its NTO effects**

1 Schäfer, M. L., Lundström, J. O. & Petersson, E. (2008) Comparison of mosquito (Diptera: Culicidae) faunas by wetland type and year in the lower River Dalälven region, Central Sweden. *Journal of Vector Ecology* **33**, 150-157.

2 Poulin, B., Lefebvre, G. & Paz, L. (2010) Red flag for green spray: adverse trophic effects of Bti on breeding birds. *Journal of Applied Ecology* **47**, 884-889.

3 Nilsson, C. & Renöfält, B. Mygg och Bti i nedre Dalälven: Utvärdering av ett vetenskapligt uppföljningsprogram. (2009).

4 DASA. http://www.slu.se/sv/fakulteter/nl/om-fakulteten/institutioner/institutionen-for-vatten-och-miljo/). .

5 Boisvert, M. & Boisvert, J. (2000) Effects of Bacillus thuringiensis var. israelensis on target and nontarget organisms: a review of laboratory and field experiments. *Biocontrol Science and Technology* **10**, 517-561.

6 Charbonneau, C. S., Drobney, R. D. & Rabeni, C. F. (1994) Effects of Bacillus-Thuringiensis var sraelensis on nontarget benthic organisms in a lentic habitat and factors effecting the efficacy of the larvicide. *Environmental Toxicology and Chemistry* **13**, 267-279.

7 Boisvert, M., Boisvert, J. & Aubin, A. (2001) Factors affecting residual dosages of two formulations of Bacillus thuringiensis subsp israelensis tested in the same stream during a 3-year experiment. *Biocontrol Science and Technology* **11**, 727-744.

8 Hannay, C. L. (1953) Crystalline inclusions in aerobic sporeforming bacteria. *Nature* **172**, 1004.

9 Poopathi, S. & Abidha, S. (2009) A Medium for the Production of Biopesticides (Bacillus sphaericus and Bacillus thuringiensis subsp israelensis) in Mosquito Control. *Journal of Economic Entomology* **102**, 1423-1430.

10 Merritt, R. W., Walker, E. D., Wilzbach, M. A., Cummins, K. W. & Morgan, W. T. (1989) A broad evaluation of bti for black fly (Diptera, Simuliidae) control in a michigan river - efficacy, carry and nontarget effects on invertebrates and fish. *Journal of the American Mosquito Control Association* **5**, 397-415.

11 Mulla, M. S., Federici, B. A. & Darwazeh, H. A. (1982) Larvicidal efficacy of Bacillus-thuringiensis serotype h-14 against stagnant-water mosquitos and its effects on nontarget organisms. *Environmental Entomology* **11**, 788-795.

12 Jackson, J. K., Horwitz, R. J. & Sweeney, B. W. (2002) Effects of Bacillus thuringiensis israelensis on black flies and nontarget macroinvertebrates and fish in a large river. *Transactions of the American Fisheries Society* **131**, 910-930.

13 Armitage, P., Cranston, P. S. & Pinder, L. C. V. 572 (Chapman and Hall, London, Glasgow, New York, Tokyo, Melbourne, Madras, 1994).

14 Liber, K., Schmude, K. L. & Rau, D. M. (1998) Toxicity of Bacillus thuringiensis var. israelensis to chironomids in pond mesocosms. *Ecotoxicology* **7**, 343-354.

15 Kondo, S., Ohba, M. & Ishii, T. (1995) Comparative susceptibility of chironomid Larvae (Dipt, Chironomidae) to bacillus-Thuringiensis serovar israelensis with

special refference to altered suspceptibility due to food difference. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie* **119**, 123-125.

16 Lundström, J. O., Brodin, Y., Schafer, M. L., Vinnersten, T. Z. P. & Ostman, O. (2010) High species richness of Chironomidae (Diptera) in temporary flooded wetlands associated with high species turn-over rates. *Bulletin of Entomological Research* **100**, 433-444.

17 Lundström, J. O., Schäfer, M. L., Petersson, E., Persson Vinnersten, T. Z., Landin, J. & Brodin, Y. (2010) Production of wetland Chironomidae (Diptera) and the effects of using Bacillus thuringiensis israelensis for mosquito control. *Bulletin of Entomological Research* **100**, 117-125.

18 Hershey, A. E., Lima, A. R., Niemi, G. J. & Regal, R. R. (1998) Effects of Bacillus thuringiensis israelensis (Bti) and methoprene on nontarget macroinvertebrates in Minnesota wetlands. *Ecological Applications* **8**, 41-60.

19 Niemi, G. J., Hershey, A. E., Shannon, L., Hanowski, J. M., Lima, A., Axler, R. P. & Regal, R. R. (1999) Ecological effects of mosquito control on zooplankton, insects, and birds. *Environmental Toxicology and Chemistry* **18**, 549-559.

20 Ahlgren, G., Goedkoop, W., Markensten, H., Sonesten, L. & Boberg, M. (1997) Seasonal variations in food quality for pelagic and benthic invertebrates in Lake Erken - the role of fatty acids. *Freshwater Biology* **38**, 555-570.

21 Vilarinhos, P. T. R. & Monnerat, R. (2004) Larvicidal persistence of formulations of Bacillus thuringiensis var. israelensis to control larval Aedes aegypti. *Journal of the American Mosquito Control Association* **20**, 311-314.

22 Boyer, S., Tilquin, M. & Ravanel, P. (2007) Differential sensitivity to Bacillus thuringiensis var. Israelensis and temephos in field mosquito populations of Ochlerotatus cataphylla (Diptera : Culicidae): Toward resistance? *Environmental Toxicology and Chemistry* **26**, 157-162.

23 Vaughan, I. P., Newberry, C., Hall, D. J., Liggett, J. S. & Ormerod, S. J. (2008) Evaluating large-scale effects of Bacillus thuringiensis var. israelensis on non-biting midges (Chironomidae) in a eutrophic urban lake. *Freshwater Biology* **53**, 2117-2128.

24 Hanowski, J. M., Niemi, G. J., Lima, A. R. & Regal, R. R. (1997) Response of breeding birds to mosquito control treatments of wetlands. *Wetlands* **17**, 485-492.

25 Poulin, B. (In Press) Indirect effects of bioinsecticides on the nontarget fauna: the Camargue experiment calls for future research. *Acta Oecologia*.

26 Mulla, M. S., Navvabgojrati, H. A. & Darwazeh, H. A. (1978) TOXICITY OF MOSQUITO LARVICIDAL PYRETHROIDS TO 4 SPECIES OF FRESHWATER FISHES. *Environmental Entomology* **7**, 428-430.

27 Ostman, O., Lundstrom, J. O. & Vinnersten, T. Z. P. (2008) Effects of mosquito larvae removal with Bacillus thuringiensis israelensis (Bti) on natural protozoan communities. *Hydrobiologia* **607**, 231-235.

28 Vinnersten, T. Z. P., Lundstrom, J. O., Petersson, E. & Landin, J. (2009) Diving beetle assemblages of flooded wetlands in relation to time, wetland type and Bti-based mosquito control. *Hydrobiologia* **635**, 189-203.

29 Länststyrelsen, V. Uppdrag om utvärdering av biologisk myggkontroll vid Nedre Dalälven (M2002/2981/Na). (ISSN 0284-8813, 2003:9, Dnr 100-10722-02, 2003).

30 Quinn, G. P. & Keough, M. J. (2002) *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, pp.

31 Osenberg, C. W., Bolker, B. M., White, J. S., St. Mary, C. M. & Shima, J. S. (2006) in *Foundations of Restoration Ecology* eds D.A. Falk, M. A. Palmer, & J.B. Zedler) Island Press, 280-302pp.

32 Downes, B. J., Barmuta, L. A., Fairweather, P. G., Faith, D. P., Keough, M. J., Lake, P. S., Mapstone, B. D. & Quinn, G. P. (2002) *Monitoring Ecological Impacts: Concepts and Practice in Flowing Waters*. Cambridge University Press, pp.

33 Downes, B. J., Hindell, J. S. & Bond, N. R. (2000) What's in a site? Variation in lotic macroinvertebrate density and diversity in a spatially replicated experiment *Austral Ecology* **25**, 128-139.

34 Searle, S. R., Casella, G. & McCulloch, C. E. (1992) *Variance Components*. Wiley, pp.

35 Gotelli, N. J. & Ellison, A. M. (2004) *A Primer of Ecological Statistics*. Sinaur, 510pp.

36 Tabachnick, B. G. & Fidell, L. S. (2001) *Multivariate Statistics*. Allyn and Bacon, pp.

37 Clarke, K. R. (1993) Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology* **18**, 117-173.

38 Gotelli, N. J. & Graves, G. R. (1996) *Null Models in Ecology*. Smithsonian Institution Press, pp.

39 Murphy, K. R., Myors, B. & Wolach, A. (2008) *Statistical Power Analysis: A Simple and General Model for Traditional and Modern Hypothesis Tests*. Routledge Academic, pp.

40 McKie, B. G. & Malmqvist, B. (2009) Assessing ecosystem functioning in streams affected by forest management: increased leaf decomposition occurs without changes to the composition of benthic assemblages. *Freshwater Biology*, doi:10.1111/j.1365-2427.2008.02150.x.

41 Lepori, F., Palm, D., Brännäs, E. & Malmqvist, B. (2005) Does restoration of structural heterogeneity in streams enhance fish and macroinvertebrate diversity? *Ecological Applications* **15**, 2060-2071.

42 Timm, N. H. & Mieczkowski, T. A. (1997) *Univariate and multivariate general linear models: theory and applications*. SAS Institute, pp.

43 Clarke, K. R. & Gorley, R. N. (2001) *Primer v5: User manual/tutorial.*, PRIMER-E, pp.

44 Growns, J. E., Chessman, B. C., Jackson, J. E. & Ross, D. G. (1997) Rapid assessment of Australian rivers using macroinvertebrates: cost and efficiency of 6 methods of sample processing. *Journal of the North American Benthological Society* **16**, 682-693.

45 Van Guelpen, L., Markle, D. F. & Duggan, D. J. (1982) An evaluation of accuracy, precision, and speed of several zooplankton sub-sampling techniques. *Journal du Conseil—Conseil International pour l'Exploration de la Mer* **40**, 226-236.

46 Lenat, D. R. & Barbour, M. T. (1994) in *Biological monitoring of aquatic ecosystems.* (ed S. L. L. A. Spacie) Lewis Publishers, 187-215pp.

47 Boisvert, M. & Boisvert, J. (1999) Persistence of toxic activity and recycling of Bacillus thuringiensis var. israelensis in cold water: Field experiments using diffusion chambers in a pond. *Biocontrol Science and Technology* **9**, 507-522.

APPENDIX I: Literature Review

Assessing the effects of the mosquito control agent Bti (Bacillus thuringiensis serovar israelensis): a literature review of Swedish and international studies.

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Assessing the effects of the mosquito control agent Bti (Bacillus thuringiensis serovar israelensis): a literature review of Swedish and international studies.

This primary aim of this contribution is to review the literature on the short- and long-term effects of Bti application on aquatic and terrestrial ecosystems, with a focus on:

(i) the mode of action and fate of Bti,
(ii) its effects on Non-target organisms,
(iii) its affects on other ecosystem properties, mediated primarily through the foodweb and
(iv) the approaches and methodologies used in assessing the effects of Bti..

This review is based initially on a detailed review by Boisvert & Boisvert (2000), which summarized the state of knowledge at that time on the effects of Bti on target and NTO. They reviewed 75 published studies, 21 from lotic (river and stream) environments, 24 in lentic (lake and other still water) environments, 27 in laboratory or artificial environments and 3 conducted under both laboratory and field conditions. In lentic habitats 90% of the studies were done using recommended dosage. Since this review, further studies have been published which are also considered here, including 14 focussing on lentic environments, and four on the fate of Bti in different habitats and under different environmental regimes.

Mode of action and the fate of Bti in treated environment

Mode of action

Bti is a member of the large family of Bacilli. It is a crystalliferous, sporeforming, aerobic bacterium closely related to *B. cereus* but differing from it by its ability to synthesize a parasporal protein crystal endotoxin generally toxic to certain insects. The association of these crystal with the toxicity of Bt to insects was established by Hannay (1953). Hannay coined the phrase `parasporal body' to describe the inclusion that lies alongside each spore. Today, the parasporal body is also termed parasporal inclusion, crystalline inclusion, toxic inclusion, crystal, protoxin or delta-ndotoxin. When ingested by a larva, the parasporal body dissolves in the alkaline gut juices, and midgut proteases cleave the protoxin, yielding the active delta-endotoxin proteins. The toxicity of the Bti parasporal body varies considerably depending on whether it is intact or solubilized and how it is assayed. When ingested, either intact or solubilized, the parasporal body is toxic to mosquitoes, black flies and several other nematocerous dipterans. But, whether ingested, injected or topically applied, the intact protoxin is not active against vertebrates. More on the mode of action can be found in the Boisvert review (Boisvert and Boisvert 2000).

The fate of Bti

The risk of long lasting (residual) toxicity of Bti in different treated habitats, including water, sediments and algal mats, has been extensively investigated (Boisvert et al. 2001; Boisvert et al. 2002; Hajaij et al. 2005; De Respinis et al. 2006; Tilquin et al. 2008). This research has yielded very little evidence for any residual toxicity of Bti. Several studies found that Bti spores accumulated in soil, plants, leaf litter and water after treatment, but these were nontoxic to NTOs and target organisms alike, even after a month's exposure (Hajaij et al. 2005; De Respinis et al. 2006). However, Boisvert et al. (1999) tested the persistence of Bti in different substrates (pond water, periphyton, sediments and vegetation) using diffusion chambers. The solid fraction of the vegetation remained toxic for up to five months (Boisvert and Boisvert 1999). Finally, the risk of bacteria recycling (Varjal de Melo-Santos et al. 2009) and developing resistance among targeted organism remains a concern (Bonin et al. 2009; Wirth et al. 2010), highlighting a need for monitoring of long-term changes in the responses of target and NTO assemblages in systems subjected to repeated treatments over many years.

Effects on NTO

More then 50 studies have been conducted on the direct toxic effect of Bti on NTOs in lab and in controlled small-scale field conditions. Overall, these experiments confirm Bti as the most highly targeted mosquito control agent currently available, and even at high dosages, Bti had little effect on most NTOs. The main exceptions are members of the Diptera suborder Nematocera, which includes mosquitos, and in particular the Chironomidae.

Chironomids – General comments

The Chironomidae (Diptera) are holometabolous insects that are commonly known as "non-biting midges" or simply "midges" (Fig. 1.1). The family is species-rich (estimated 10 000+ species world wide) and ecologically diverse, with larvae occurring in fresh and salt water and on land across all altitudes and most latitudes, including Antarctic regions (Cranston 1995a). The family encompasses all main feeding modes, including generalist particulate detritivores, predators, algal grazers, ectoparasites, and leaf/wood miners (Berg 1995; Pinder 1995b). The larval stage is concerned with resource acquisition, and is longest in duration (lasting from weeks to months, and sometimes years). The adults look similar to their close relatives the mosquitoes (Culicidae), blackflies (Simuliidae) and sandflies (Ceratopoginidae). However, unlike those families, adult chironomids (with the possible exception of some primitive species) are not bloodsuckers; rather, feeding is limited to the occasional sipping of nectar or honeydew to supplement flight energy reserves in a few species. The adults are short lived (a few days at most), with the bulk of stored resources channelled into reproduction (Armitage 1995a). Chironomids are a key component of food-webs in both aquatic and terrestrial environments.

The susceptibility of chironomids to Bti in field studies is often not clear-cut, and depends on the dosage and application method, and also the method used to sample the chironomids. Nevertheless, there are multiple documented cases of chironomid mortality, and reductions in chironomid density during application of *Bti* targeting mosquitos (reviewed in Boisvert & Boisvert, 2000). Across muliple studies, the susceptibility of chironomid larvae to Bti could be between 15 to 75 times less than mosquito larvae. However, some species seem very susceptible; for example *Chironomus kiiensis, C. yoshimatsui* and *Paratanytarsus* sp., for which 17 µg/ml of *Bti* resulted in high (70-100%) and rapid mortality (within 2 days) (Kondo et al. 1995), and high dosages, whether accidental or deliberate, certainly have potential to impact chironomid populations. Additionally, Boisvert & Boisvert (2000) made the remark that 11 out of 23 studies (Eight in lotic environments, one in lentic environments and two in the laboratory) in which chironomids were affected, Bti was applied at operational dosages, that is, at levels intended to affect mosquitos, and not excessively high. Nine of these 11 studies were done in field conditions. Overall, nearly 40% of the studies (9 out of 23) reporting an effect on chironomid populations were done using actual operating conditions.

It would therefore appear that the feeding behavior of certain species of chironomids could make them highly susceptible to Bti even in conditions where `operational dosages' would be used for treatment of mosquito breeding sites (Boisvert and Boisvert 2000).

Chironomidae species susceptible to Bti can be found in Database 1 (at the end of this review) as well as in Boisvert & Boisvert (2000) review.

Long term field studies on the effects of Bti on NTO

Detection of long term, cumulative effects of repeated Bti applications on an ecosystem requires monitoring over a longer time period (interannual). Here, "long-term" is used to refer to studies longer then 2 years. The lack of studies lasting over interannual timescales is repeatedly remarked upon in the Bti literature. Only a few long-term studies on the effects of Bti in the field have been conduced. Most publications of studies longer then 2 years result from two monitoring programs: (i) from Wright county Minnesota, USA (conducted from 1988 to 1993) and (ii) from irregularly flooded wetlands of the River Dalalven in Sweden (conducted from 2002 to 2007). Additional long term studies come from France and Wales.

(i) Wright County, Minnesota

Hershey and colleagues (Hershey et al. 1995; Hershey et al. 1998) studied the effect of Bti on benthic macroinvertebrate communities of 27 wetland ecosystems in Wright county Minnesota, USA. Following 3 years of preliminary study and sampling they treated 9 wetlands with Bti, with left 9 wetlands untreated. They repeated this treatment for 3 years. For the first 2 years of treatment, no effect of Bti on NTO was detected, However, in the third year significant effects on the densities, richness and dominance of NTO, predominantly nematoceran Diptera, were detected.

Over the three years, at least 179 genera of aquatic insects representing seven orders were sampled from the study area. Of these, 101 were Diptera, and 51 of the Diptera were Chironomidae.

Insect density was lower in the Bti treated areas (63% lower overall). The authors further mentioned that while the control sites showed seasonal increases in insect abundance, Bti treated sites did not.
Total dipteran density was similar on Bti and control sites in 1991, but 62% and 82% lower in 1992 and 1993, respectively.
Nematocera density in Bti treated sites was similar to control sites during pretreatment years and during 1991, the first year of larvicide treatment. However, in1992 and 1993, Bti sites had one-third to one-sixth the number of Nematocera, respectively, as controls. The Nematocera were overwhelmingly dominated by Chironomidae, which comprised up to 90% of the nematoceran individuals sampled on most sites, and 60% of the total insects sampled. Tipulidae were also affected in a similar way as chironomidae. Ceratopogonids were affected only in the third year. Within the non-insect group, no seasonal effects of Bti were observed for any of the abundant taxa.
Insect richness - Total insect richness, expressed as number of genera, was reduced in Bti sites by one-third to two-thirds that of control sites during the 1992 and 1993 seasons, respectively.
Dominance - Bti sites showed a greater tendency to be dominated by one or a few genera than did control sites during 1992 and 1993. This represents a decline in the species evenness dimension of biodiversity.

Since the worst affected NTO were predominantly Chironomidae, which are important food items for many wetland predators, the authors suggested that their failure to develop in treated sites might represent a substantial difference in the function, as well as the structure, of the insect community. Niemi et al.(1999) addressed this in a study of the same wetlands where zooplankton and breeding birds were additionally sampled. They found the same effect of Bti treatment on nematoceran insects, but found no effects on zooplankton or red-winged blackbirds. The authors suggested that their experimental design was not adequate for assessing effects on breeding birds, which thus required further study. Hanowski et al. (1997) had earlier suggested that local bird species might often be able to find immediate substitutes for the prey lost following Bti treatment. However, because of lower aquatic insect densities in mid- to late-summer, other parts of the avian life cycle such as late summer survival, dispersal of young birds, or migrating birds, may be more affected by mosquito control treatments. These aspects were not directly assessed during the Minnesota studies (Hanowski, Niemi et al. 1997, (Niemi et al. 1999).

(ii) River Dalälven, Sweden

Another long term study was conducted at irregularly flooded wetlands of the River Dalalven flood-plains in central Sweden during 6 years starting from 2002 by Lundström and colleagues (Lundstrom et al. 2010; Lundstrom et al. 2010). They sampled 6 wetlands, 3 of them were treated and three not. Sampling was conducted via 4 insect emergence traps deployed continuously at each location and sampled weekly, generally from May to September. They found no evidence for negative Bti treatment effects on the abundance or species richness of emerging NTOs, including chironomids. Species turnover between years was larger in experimental wetlands than in reference wetlands, and more chironomid species were found in experimental wetlands than in reference wetlands. The authors suggested that this reflected a higher random colonisation from a regional species pool in the Bti treated wetlands, rather than some species responding directly to the Bti treatment.

Further studies focused on other NTO groups from this long-term monitoring program showed either minimal, or even positive, results from the Bti application. Vinnersten et al. (2009) studied the effects of Bti treatment around Lake Färnebofjärden in the River Dalalven flood-plains on the diving beetle assemblages. They found the only effect of Bti-treatment against flood-water mosquito larvae, potential food for the predatory dytiscids, was a slight increase in abundance of the medium-sized dytiscid species. Östman & Lundström (2008) reported that the densities of heterotrophic protozoans was on an average 4.5 times higher in wetland areas treated with Bti than in control areas. In addition, the taxonomic richness of heterotrophic protozoans increased on an average of 60% in areas with Bti application compared to control areas.

Further research is needed to assesses the ecological implications of these findings, which are not clear. Increased productivity within one food web compartment may have positive or negative consequences for other compartments, depending on if and how they transfer through the trophic web.

With only three wetlands of each type (treated and reference), the statistical power in these studies for detecting more subtle, potentially "early warning", effects of Bti was low, further compounded by the very high variability in their data, reflected particularly in the low proportion of variance explained in their statistical models. However, the lack of strong effects of Bti in the Dalälven contrast with results from Minnesota (see above) and France (see below), and demonstrates that consequences of Bti application may depend strongly on characteristics of the treated system, and so are difficult to predict a-priori.

(iii) Additional long-term studies

Long term research on breeding birds was conducted by Poulin et al. (Poulin et al. 2010; Poulin In Press). Nestling diet was assessed at one control and one Bti treated site in June 2006 before the first Bti application, and continued to 2009, with an equal number of control and treated sites increasing from 1 (2007) to 2 (2008) and 3 (2009). They found that house martins had a lower intake of Nematocera at treated sites, and a higher intake of flying ants. The birds also showed lower foraging rates at treated sites, suggesting a lower food availability, with ants acting as a substitute for Nematocera. Finally, the study also revealed a decrease in fledging success at the treated sites, which most likely explained by chick starvation. While the authors did not directly quantify the effects of Bti on the flying insects, they suggested that these differences in dietary composition related to a locally lower abundance of these Bti-sensitive insects, and thus demonstrate an effect of Bti treatment ramifying through the food web. A newer publication from this program further reports on negative effects on intermediate invertebrate predators (spiders and dragonflies) associated with reed habitats (Poulin In Press).

At Cardiff Bay, in south western Britain, Vaughan et al. (2008) conducted a two year study in a 200 ha artificial water body. They had 3 Bti treatment and 3 control localities, from which they sampled benthic Chironomidae. Two sets of experiments were conducted. In the first, they had 3 treated and 3 control localities, but sampled only 7 sites within each locality. In the second experiment they had only one control area but they sampled 20 sites. The first experiment yielded evidence of weak Bti effects over the course of 2 years, including six Bti treatments. In contrast, the second experiment showed markedly stronger effects, with a 35% decrease in chironomid larva at treated sites comparing to 10-23% increase in control sites. These results highlight the potential importance of sampling regime for the capacity to detect effects.

Short term field studies on the effect on non-targeted organism (1999 to 2010)

It is more common to find short term (less than 2 years from Bti treatment) studies of Bti effects in the literature. Older work of this nature is well-summarised in Boisvert & Boisvert (2000). More recent (1999-2010) conducted in the field is summarized here.

A qualitative and quantitative population survey of immature and adult Chironomidae was conducted for 1 year in wetlands at a country club in northeast Florida, USA (Ali et al. 2008). They took benthic samples for larva and light trap samples for adults. They did not mention the effect of treatments on adults but 47-52% reduction in the total larval chironomid population was reported.

A salt marsh in Moreton Bay, a large, subtropical, estuarine embayment in southeast Queensland, Australia, was sampled by Russell et al. (2009). They sampled terrestrial plots with pit-fall traps and aspirators, the ephemeral pools were sampled using a sweep net. They found that the effects of Bti on the community composition of terrestrial and aquatic habitats were extremely variable from place to place, but were generally short-term. However they conclude that more work is needed to determine if there are chronic (non-lethal) impacts that may lead to longer-term effects on saltmarsh communities.

Duchet et al. (2008) conducted a study to evaluate the effect of Bti on a natural population of Daphnia pulex in a microcosms placed in shallow temporary oligohaline marsh located in Le Tour du Parc, France. They assessed the effect of a single Bti treatment after 2,4,7,14,21 days. Bti had no effect on D. pulex population survival, confirming a number of previous studies (reviewed in Boisvert and Boisvert, 2000).

Two studies were conducted during the late summers of 2004 through 2006 at Benton Lake National Wildlife Refuge near Great Falls, MT, USA. (Davis and Peterson 2008). The first experiment assessed the acute impacts on nontarget aquatic and terrestrial arthropods after a single application and the second experiment assessed the longer-term impact after multiple applications. In both experiments they found few, if any, deleterious effects in treated ponds.

Summary of indirect Bti effects, mediated through the food-web

Regardless of its direct toxic effects on NTO, Bti still has potential to affect the functioning of both aquatic and terrestrial environments by reducing the biomass of mosquitos, an important food source. Additional effects on chironomid NTO, themselves a key component of aquatic and terrestrial trophic webs, would only condound this. To date, there have been very few studies on the effect of Bti on the functioning and stability of foodwebs. Research of this nature has tended to focus on top consumers. Research on birds has been mentioned previously, but is presented here again as part of a summary of food-web effects.

Effect on fish – no effects of Bti applications on fish communities have been detected (Brown et al. 2002; Jackson et al. 2002).

Effect on birds - Long-term field studies with Bti used to control mosquitoes in wetlands in Minnesota, USA (discussed above) indicated that Bti has no effect on red wing black bird populations (Hershey et al. 1998; Niemi et al. 1999), or on 19 additional bird species (Hanowski et al. 1997). However, the authors regarded their research design as inadequate and emphasized a need for further research. More recently, a long-term study by Poulin et al. (2010) showed clear differences in the foraging and fledging success of breeding birds between Bti-treated and wetland ecosystems. Finally, it has been suggested that the widespread use of Bti in North America may be a contributing factor in the continent-wide decline of insectivorous birds (Gilbert 2010).

Effect on other invertebrates – The use of Bti in southern France has been associated with reductions in the densities of intermediate invertebrate predators (spiders and dragonflies) associated with reed habitats, presumably reflecting reduced quantities of mosquito prey in their diets. There is some evidence that the use of Bti in the Swedish Dalälven is associated with increases in medium size Dytiscid beetles, though this may have been a sampling artefact (Vinnersten et al. 2009; Poulin In Press).

Effect on algae and protozoans -Treatment of microcosms with Bti resulted in good control of immature mosquito populations but also suppressed the growth of two algal species, *Closterium* sp. and *Chlorella* sp. (Su and Mulla 1999). The reduction in algal populations resulted in reduced photosynthesis, lower water turbidity and oxygen concentrations in the treatments compared to controls, especially during the hot season. Boisvert and Boisvert (2000) in their review on Bti effects on nontarget organisms suggest that the declines in insect biomass observed by Hershey et al. (1998) and Niemi et al. (1999) may be explained by a reduction in algal biomass which in turn would have a major impact on the entire food web.

Östman & Lundström (2008) reported that the densities of heterotrophic protozoans was on an average 4.5 times higher in wetland areas treated with Bti than in control areas. In addition, the taxonomic richness of heterotrophic protozoans increased on an average of 60% in areas with Bti application compared to control areas.

Further research is needed to assesses the ecological implications of these findings, which are not clear. Increased productivity within one food web compartment may have positive or negative consequences for other compartments, depending on if and how they transfer through the trophic web.

Comparison of studies: Dosages, experimental design, and sampling methodology

Boisvert and Boisvert (2000) early in their review remark that a major problem encountered in reviewing the literature is the inconsistent reporting of the specifics of the Bti dosages and preparations used in the various mosquito control programs. Sometimes, studies reported using the “recommended dosage”, but this can vary among jurisdictions (for example (Duchet et al. 2008; Vinnersten et al. 2009)), while others, including studies where some of the strongest effects were observed (Poulin et al. 2010), did not mention the dosage used at all (Russell et al. 2009; Lundstrom et al. 2010; Poulin et al. 2010). This makes it difficult to assess if, for example, differences in the effects of Bti observed in different studies relate to differences in the application of the Bti treatment itself. Accordingly, a priority for future biomonitoring should be the careful recording of the operational dosage of Bti used.

Some of the variation in the reported effects of Bti on NTO organisms and other ecosystem properties undoubtedly reflects differences in the systems studied. For example, it is possible that the lack of strong effects observed in the Dalälven might reflect the high proportion of terrestrial chironomids in that system (Lundström et al. 2010), and differences in the susceptibilities of terrestrial vs aquatic taxa certainly needs more research. However, the contrasting results from the long term studies might also reflect differences in sampling design and protocols.

The study which reported the strongest effects of Bti on NTO was that by Hershey and colleagues, from Minnesota (Hershey et al. 1995; Hershey et al. 1998). This may partly reflect the extent of Bti treatments, with six applications made at each treatment site from early spring until mid- to late summer repeated at 3- wk intervals or after a rainfall of 0.78 cm, whichever occurred first. However, Hershey et al’s (1998) sampling desing had good spatio-temporal replication, limiting the possibilities for Type II statistical error, which occurs when insufficient replication or excessive noise in the data obscure a real effect (Quinn and Keough 2002). Hershey et al. (1998) were able to monitor some of their wetlands for 3 years prior to the first Bti application, allowing for an extensive pre-disturbance data set. Additionally, Hershey et al. had reasonably extensive “whole-wetland replication” with 9 treated and 9 controls sites for each year. The availability of both predisturbance data and replicated sites allows the use of a complete Before-After Control-Impact (BACI) analysis (Downes et al. 2002).

Interestingly, all studies which have reported effects of BTI on NTO chironomids have sampled chironomids in the larval stage from the benthos, whereas the Swedish Dalälven research program has relied on emergence traps, to sample emerging adult insects. However, caution is needed in generalising from this observation, due to the low number of studies overall. Emergence traps appear to be the most tractable option for sampling the extensive Dalälven wetlands, and there seems no in principal reason why they should not be less effective in detecting differences between treated and untreated wetlands, if they are effectively sampling a high proportion of the emerging insects. Nevertheless, the effectiveness of the emergence traps for sampling the chironomids relative to benthic methodologies requires further assessment.

It is possible that the contrasting results from the Dalälven and Minnesota wetlands reflect differences in environmental characteristics and the composition of the biota. If sampling protocol is important, it seems more likely to relate to the extent of sampling rather than the approach used.

In Dalälven, four emergence traps were sampled from 6 wetlands, comparied with eight core tube (19.6 sqcm area) samples from 19 wetlands in the Minnesota study (Hershey et al. 1998). The multi-year study study by Vaughan et al. (2008) are indicative of the importance of the size of the sampling effort and chosen methods. When they sampled 4 samples in each site they got no effect of Bti treatment on chironomids larvae but when they sampled 20 samples in each site they observed 35% decrease in treated site comparing to 10% increase in control sites.

Very few studies have been designed specifically to assess the effects of mosquito control on other trophic levels, and none have considered the effects of Bti on ecosystem processes related to nutrient flux (eg: productivity, nutrient uptake rates, detrital decomposition etc). Research on such topics may have to consider additional factors specific to the organisms or processes under study. For example, effects of weather and predation can have more important short-term iinfluences on bird communities than a mosquito control agent applied during the main mosquito hatching period, necessitating a longer study period to detect the effects of a reduced aquatic food supply on parameters such as late summer survival or dispersal of young birds (Hanowski et al. 1997). Similarly, a study of the effects of Bti on ecosystem processes would need to consider the longer-term fate of the nutrients and energy retained on the floodplain in he biomass of decaying mosquito larval corpses.

REFERENCES

Ali, A., R. J. Lobinske, et al. (2008). "Population survey and control of Chironomidae (Diptera) in wetlands in northeast florida, USA." *Florida Entomologist* **91**(3): 446-452.

Armitage, P. D. (1995a). Behaviour and ecology of adults. *The Chironomidae: Biology & Ecology of Non-Biting Midges*. P. D. Armitage, P. S. Cranston and L. C. V. Pinder. London, Chapman & Hall**:** 194-224.

Berg, M. B. (1995). Larval food and feeding behaviour. *The Chironomidae: Biology & Ecology of Non-Biting Midges*. P. D. Armitage, P. S. Cranston and L. C. V. Pinder. London, Chapman & Hall**:** 136-168.

Boisvert, M. and J. Boisvert (1999). "Persistence of toxic activity and recycling of Bacillus thuringiensis var. israelensis in cold water: Field experiments using diffusion chambers in a pond." *Biocontrol Science and Technology* **9**(4): 507-522.

Boisvert, M. and J. Boisvert (2000). "Effects of Bacillus thuringiensis var. israelensis on target and nontarget organisms: a review of laboratory and field experiments." *Biocontrol Science and Technology* **10**(5): 517-561.

Boisvert, M., J. Boisvert, et al. (2001). "Factors affecting residual dosages of two formulations of Bacillus thuringiensis subsp israelensis tested in the same stream during a 3-year experiment." *Biocontrol Science and Technology* **11**(6): 727-744.

Boisvert, M., J. Boisvert, et al. (2002). "Influence of stream profile and abiotic factors on the performance and residual dosages of two commercial formulations of Bacillus thuringiensis subsp israelensis during a 2-year experiment." *Biocontrol Science and Technology* **12**(1): 19-33.

Bonin, A., M. Paris, et al. (2009). "Candidate genes revealed by a genome scan for mosquito resistance to a bacterial insecticide: sequence and gene expression variations." *Bmc Genomics* **10**.

Brown, M. D., J. Carter, et al. (2002). "Pulse-exposure effects of selected insecticides to juvenile Australian crimson-spotted rainbowfish (Melanotaenia duboulayi)." *Journal of Economic Entomology* **95**(2): 294-298.

Cranston, P. S. (1995a). Introduction. *The Chironomidae: Biology & Ecology of Non-Biting Midges*. P. D. Armitage, P. S. Cranston and L. C. V. Pinder. London, Chapman & Hall**:** 1-7.

Davis, R. S. and R. K. D. Peterson (2008). "Effects of single and multiple applications of mosquito insecticides on nontarget arthropods." *Journal of the American Mosquito Control Association* **24**(2): 270-280.

De Respinis, S., A. Demarta, et al. (2006). "Molecular identification of Bacillus thuringiensis var. israelensis to trace its fate after application as a biological insecticide in wetland ecosystems." *Letters in Applied Microbiology* **43**(5): 495-501.

Downes, B. J., L. A. Barmuta, et al. (2002). *Monitoring Ecological Impacts: Concepts and Practice in Flowing Waters*. Cambridge UK, Cambridge University Press.

Duchet, C., M. Larroque, et al. (2008). "Effects of spinosad and Bacillus thuringiensis israelensis on a natural population of Daphnia pulex in field microcosms." *Chemosphere* **74**(1): 70-77.

Gilbert, N. (2010). "Mosquito spray affects bird reproduction." *Nature News* **doi:10.1038/news.2010.296**.

Hajaij, M., A. Carron, et al. (2005). "Low persistence of Bacillus thuringiensis serovar israelensis spores in four mosquito biotopes of a salt marsh in southern france." *Microbial Ecology* **50**(4): 475-487.

Hannay, C. L. (1953). "Crystalline inclusions in aerobic sporeforming bacteria." *Nature* **172**: 1004.

Hanowski, J. M., G. J. Niemi, et al. (1997). "Response of breeding birds to mosquito control treatments of wetlands." *Wetlands* **17**(4): 485-492.

Hershey, A. E., A. R. Lima, et al. (1998). "Effects of Bacillus thuringiensis israelensis (Bti) and methoprene on nontarget macroinvertebrates in Minnesota wetlands." *Ecological Applications* **8**(1): 41-60.

Hershey, A. E., L. Shannon, et al. (1995). "Effects of methoprene and Bti (bacillus-thurngiensis var israelensis) on nontarget insects." *Hydrobiologia* **308**(3): 219-227.

Jackson, J. K., R. J. Horwitz, et al. (2002). "Effects of Bacillus thuringiensis israelensis on black flies and nontarget macroinvertebrates and fish in a large river." *Transactions of the American Fisheries Society* **131**(5): 910-930.

Kondo, S., M. Ohba, et al. (1995). "Comparative susceptibility of chironomid Larvae (Dipt, Chironomidae) to bacillus-Thuringiensis serovar israelensis with special refference to altered suspceptibility due to food difference." *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie* **119**(2): 123-125.

Lundstrom, J. O., Y. Brodin, et al. (2010). "High species richness of Chironomidae (Diptera) in temporary flooded wetlands associated with high species turn-over rates." *Bulletin of Entomological Research* **100**(4): 433-444.

Lundström, J. O., Y. Brodin, et al. (2010). "High species richness of Chironomidae (Diptera) in temporary flooded wetlands associated with high species turn-over rates." *Bulletin of Entomological Research* **100**(4): 433-444.

Lundstrom, J. O., M. L. Schafer, et al. (2010). "Production of wetland Chironomidae (Diptera) and the effects of using Bacillus thuringiensis israelensis for mosquito control." *Bulletin of Entomological Research* **100**(1): 117-125.

Niemi, G. J., A. E. Hershey, et al. (1999). "Ecological effects of mosquito control on zooplankton, insects, and birds." *Environmental Toxicology and Chemistry* **18**(3): 549-559.

Pinder, L. C. V. (1995b). The habitats of chironomid larvae. *The Chironomidae: Biology & Ecology of Non-Biting Midges*. P. D. Armitage, P. S. Cranston and L. C. V. Pinder. London, Chapman & Hall**:** 107-135.

Poulin, B. (In Press). "Indirect effects of bioinsecticides on the nontarget fauna: the Camargue experiment calls for future research." *Acta Oecologia*.

Poulin, B., G. Lefebvre, et al. (2010). "Red flag for green spray: adverse trophic effects of Bti on breeding birds." *Journal of Applied Ecology* **47**(4): 884-889.

Poulin, B., G. Lefebvre, et al. (2010). "Red flag for green spray: adverse trophic effects of Bti on breeding birds." *Journal of Applied Ecology* **47**: 884-889.

Quinn, G. P. and M. J. Keough (2002). *Experimental Design and Data Analysis for Biologists*. Cambridge, UK, Cambridge University Press.

Russell, T. L., B. H. Kay, et al. (2009). "Environmental effects of mosquito insecticides on saltmarsh invertebrate fauna." *Aquatic Biology* **6**(1-3): 77-90.

Su, T. and M. S. Mulla (1999). "Microbial agents Bacillus thuringiensi s ssp. israelensis and Bacillus sphaericus suppress eutrophication, enhance water quality, and control mosquito in microcosms." *Environmental Entomology* **28**: 761- 767.

Tilquin, M., M. Paris, et al. (2008). "Long Lasting Persistence of Bacillus thuringiensis Subsp israelensis (Bti) in Mosquito Natural Habitats." *Plos One* **3**(10).

Varjal de Melo-Santos, M. A., A. P. de Araujo, et al. (2009). "Long lasting persistence of Bacillus thuringiensis serovar. israelensis larvicidal activity in Aedes aegypti (Diptera: Culicidae) breeding places is associated to bacteria recycling." *Biological Control* **49**(2): 186-191.

Vaughan, I. P., C. Newberry, et al. (2008). "Evaluating large-scale effects of Bacillus thuringiensis var. israelensis on non-biting midges (Chironomidae) in a eutrophic urban lake." *Freshwater Biology* **53**(10): 2117-2128.

Vinnersten, T. Z. P., J. O. Lundstrom, et al. (2009). "Diving beetle assemblages of flooded wetlands in relation to time, wetland type and Bti-based mosquito control." *Hydrobiologia* **635**(1): 189-203.

Wirth, M. C., W. E. Walton, et al. (2010). "Evolution of resistance to the Bacillus sphaericus Bin toxin is phenotypically masked by combination with the mosquitocidal proteins of Bacillus thuringiensis subspecies israelensis." *Environmental Microbiology* **12**(5): 1154-1160.

Database 1: List of non-target organisms susceptible to Bti

Diptera: Anisopodidae

Sylvicola fenestralis. Houston et al. 1989; Coombs et al. 1991

Diptera: Chironomidae

Chironomus attenuatus. Garcia et al. 1982
C. crassicaudatus. Ali et al. 1981
C. decorus. Mulla et al. 1990; Rodcharoen et al. 1991
C. fulvipilus. Rodcharoen et al. 1991
C. kiiensis. Kondo et al. 1995
C. riparius. Cilek and Knapp 1992
C. tepperi. Treverrow 1985
C. yoshimatsui. Kondo et al. 1992; 1995
Dicrotendipes pelochloris. Kondo et al. 1995
Dicrotendipes sp. Rodcharoen et al. 1991
Glyptotendipes paripes. Ali et al. 1981
G. tokunagai. Kondo et al. 1995
Hydrobaenus kondoi. Kondo et al. 1992
Limnophyes minimus1. Houston et al. 1989
Metriocnemus hygropetricus Houston et al. 1989
Orthocladius fuscimanus. Houston et al. 1989
Paralauterborniella elachista. Rodcharoen et al. 1991
Paratanytarsus sp. Kondo et al. 1995
P. grimmii. Kondo et al. 1995
Pentapedilum tigrinum. Kondo et al. 1995
Rheotanytarsus spp. Molloy 1992; Merritt et al. 1989
Rheotanytarsus fuscus. Palmer 1993
Stictochironomus akizukii. Kondo et al. 1995
Tanytarsus spp. Ali et al. 1981
Tokunagayusurika akamusi1. Kondo et al. 1992

Diptera: Glossinidae

Glossina pallidipes1 van-der Geest et al. 1982

Diptera: Muscidae

Haematobia irritans. Temeyer 1984

Diptera: Phlebotominae

Phlebotomus papatasi. Barjac et al. 1981; Yuval and Warburg 1989
P. argentipes. Yuval and Warburg 1989
P. perniciosus. Yuval and Warburg 1989

Diptera: Phoridae

Megaselia halterata. Kiel 1991

Diptera: Psychodidae

Lutzomyia longipalpis. Barjac et al. 1981; Yuval and Warburg 1989
Psychoda alternata. Houston et al. 1989
P. severini. Houston et al. 1989

Diptera: Sciaridae

Bradysia coprophila. Osborne et al. 1985
Bradysia spp. Harris 1993
Lycoriella mali. Cantwell and Cantelo 1984; Kiel 1991

Diptera: Simuliidae

Austrosimulium laticorne. Chilcott et al. 1983
A. multicorne. Chilcott et al. 1983
Cleitosimulium argenteostriatum [=S. argenteostriatum]. Car and Kutzer 1988
Cnephia pecuarum. Atwood et al. 1992
Cnetha verna [=Simulium vernum]. Olejnicek, 1986
Eusimulium vernum [=Simulium vernum]. Colbo and O.Brien 1984
Odagmia ornata [=Simulium ornatum]. Olejnicek 1986; Deschle et al. 1988
Prosimulium mixtum. Colbo and O.Brien 1984
Prosimulium tomosvaryi. Olejnicek 1986
Simulium aokii. Nakamura et al. 1985
S. aureum. Barton et al. 1991
S. chutteri. Palmer 1993; Palmer et al. 1996
S. damnosum. Guillet et al. 1982; Palmer 1993
S. gariepense. Palmer 1993
S. goeldii. Habib 1983

S. japonicum. Nakamura et al. 1985
S. jenningsi. Merritt et al. 1989
S. mcmahoni. Palmer 1993
S. monticola. Car and Kutzer 1988
S. noelleri. Car and Kutzer 1988
S. notiale. Barton et al. 1991
S. ochraceum. Undeen et al. 1981
S. pertinax. Araujo et al. 1990; Andrade et al. 1991; Castello-Branco et al. 1992
S. posticatum. Welton and Ladle 1993
S. pugetense. Molloy and Jamnback 1981
S. reptans. Car and Kutzer 1988; Coupland 1993
S. rorotaense. Habib 1983
S. tuberosum complex. Molloy and Jamnback 1981; Car and Kutzer 1988; Merritt et al. 1989
S. uchidai Nakamura et al. 1985
S. variegatum Car and Kutzer 1988
S. variegatum group (S. variegatum/S. argyreatum). Coupland 1993
S. venustum/S. verecundum complex. Molloy and Jamnback 1981; Barton et al. 1991
S. vernum. Riley and Fusco 1990
S. vittatum. Frommer et al. 1980; Merritt et al. 1989
Stegopterna mutata. Colbo and O.Brien 1984

Diptera: Tabanidae

Tabanus triceps. Saraswathi and Ranganathan 1996
Diptera: Tephritidae
Ceratitis capitata. El-Sebae and Komeil 1990

Diptera: Tipulidae

Tipula paludosa. Smits and Vlug 1990
T. oleracea. Smits et al. 1993; Smits and Vlug 1990

Acari: Argasidae (ticks)
Argas persicus. Hassanain et al. 1997

Acari: Ixodidae
Hyalomma dromedarii. Hassanain et al. 1997

Acari: Pyroglyphidae (mites)

Dermatophagoides pteronyssinus. Saleh et al. 1991

Mallophaga: Menoponidae

Menopon gallina. Lonc and Lachowicz 1987

Nematoda: Meloidogynidae

Meloidogyne incognita. Sharma 1994

Database 2: List of non-target organisms **not** susceptible to Bti

ACARI

Hydrachnella sp. Becker and Margalit 1993
Hydracarina sp. Mite. Beck 1982
Hydrachna sp. Mite.

AMPHIBIANS

Hylo regilla. tree frog tadpole. Abbott Laboratories; Garcia et al. 1980
Bufo sp. toad tadpole
Bufo bufo Becker and Margalit 1993
Bufo viridis
Bufo calamita
Taricha torosa. California newt. Abbott Laboratories; Garcia et al. 1980
Rana temporaria Paulov 1985a & b
Triturus alpestris Becker and Margalit 1993
Triturus vulgaris
Triturus cristatus
Bombina variegata
Rana esculenta

FISH

Gambusia affinis
Lucania parva
Gasterosteus wheatlandi
Lepomis macrochirus
Abbott Laboratories; Garcia et al. 1980 Christensen 1990a
Salvelinus fontinalis
Salmo trutta
Oncorhynchus mykiss
Wipfli et al. 1994; Wipfli et al. 1994; Christensen 1990b
Pseudomugil signifer Pacific blue-eye fish. Brown et al. 1998
Poecilia reticulate. larvivorous fish. Mittal et al. 1994
Tilapia nilotica. Lebrun and Vlayen 1981
Esox lucius Becker and Margalit 1993
Cyprinus carpio
Perca fluviatilis
Ambloplites rupestris. rock bass. Merritt et al. 1989
Epiplatys sp. killifish Beck 1982
Cyprinoidei goldfish
Cyprinodon variegatus sheephead minnow Christensen 1990c

CRUSTACEANS

Orconectes limosus. Crayfish. Becker and Margalit 1993

Amphipoda

Gammaridae sp. scuds Abbott Laboratories; Garcia et al. 1980
Hyalella azetaca sideswimmer Abbott Laboratories; Garcia et al. 1980
Gammarus duebeni Salt-marsh Roberts 1995
Gammarus pulex crustaceans Becker and Margalit 1993
Hyallela azteca Gharib and Hilsenhoff 1988

Palaemonidae

Leander tenuicornis

Palaemonetes varians

Brown et al. 1996; Roberts 1995

Decapoda

Hemigrapsus sp. purple shore crab Abbott Laboratories; Garcia et al. 1980

Anostraca

Artemia salina fairy shrimp Abbott Laboratories; Garcia et al. 1980

Conchostracans

Eulimnadia texana clam shrimp Mulla 1990

Cladocera

Simocephalus vetulus water flea Abbott Laboratories; Garcia et al. 1980

Daphnia Ali 1981

Daphnia magna Lebrun and Vlayen 1981

Daphnia pulex. Becker and Margalit 1993

Moina rectirostris Mulla 1990

Moina macrocopa Beck 1982

Chirocephalus grubei anostacan snail Becker and Margalit 1993

Ostracoda

Ostracoda. Becker and Margalit 1993; Ali 1981

Cypridae sp. seed shrimp Abbott Laboratories; Garcia et al. 1980

Copepoda

Macrocyclops sp. copepods Abbott Laboratories; Garcia et al. 1980

Macrocyclops albidus

Mesocyclops longisetus

M. ruttneri

Acanthocyclops vernalis

Marten et al. 1993

Cyclops spp. Ali 1981; Beck 1982

Cyclops strenuus Becker and Margalit 1993

Isopoda

marine sow bug Abbott Laboratories; Garcia et al. 1980;

Knepper and Walker 1989

Asellus aquaticus Becker and Margalit 1993

INSECTS

Ephemeroptera

Baetis sp. Ali 1981

Callibaetis sp. mayfly nymphs Abbott Laboratories; Garcia et al. 1980

Callibaetis pacificus Mulla 1990

Stenonema Merrit et al. 1989

Cloeon dipterum Becker and Margalit 1993

Leptoplebia sp. Beck 1982

Caenis lactea

Ephemera danica

Odonata

Ischnura sp.

Anax sp.

damselfly nymphs Abbott Laboratories; Garcia et al. 1980

Erythemis simplicicollis Painter et al. 1996

Tarnetrum corruptum

Enallagma civile

Aly and Mulla 1987
Ischnura elegans Becker and Margalit 1993
Symetrium striolatum
Orthetrum brunneum
Cordulia sp. dragonfly nymph Beck 1982

Hemiptera

Trichocorixa reticulata
Hesperocorixa leavigata
Trichocorixa
Corixidae water boatmen Abbott Laboratories; Garcia et al., 1980
Bueona scimitra backswimmer Abbott Laboratories; Garcia et al. 1980
Notonecta kirby backswimmer Abbott Laboratories; Garcia et al. 1980
Notonecta undulata
Notonecta glauca
Buenoa antigone backswimmers Aly and Mulla 1987
Beck 1982; Olejnicek and Maryskova 1986
Quiroz-Martinez et al. 1996
Pleidae pygmy backswimmer Abbott Laboratories; Garcia et al. 1980
Micronecta meridionalis Becker and Margalit 1993
Sigara lateralis water bug Beck 1982; Becker and Margalit 1993
Ranatra sp. Beck 1982
Ilyocoris cimicoides Becker and Margalit 1993
Anisops varia

Heteroptera

Plea leachi Becker and Margalit 1993

Coleoptera

Tropisternus salsamentus scavenger and Abbott Laboratories; Garcia et al. 1980
predaceous water beetle
Peltodytes edentulus,
Haliplus immaculicollis
Hydroporus undulatus
Laccophilus maculosus Gharib and Hilsenhoff 1988
Tropisternus sp. Abbott Laboratories; Garcia et al. 1980
Dytiscidae Abbott Laboratories; Garcia et al. 1980
Hyphydrus ovatus
Guignotus pusillus
Coelambus
impressopunctatus
Hygrotus inaequalis
Hydroporus palustris
Ilybius fuliginosus
Rhantus pulverosus
Rhantus consputus
Hydrobius fuscipes
Anacaena globulus
Hydrophilus caraboides
Berosus signaticollis
Becker and Margalit 1993

Bombyx mori Nataraju et al. 1993
Baeosus sp.
Coelambus sp.
Gyrinidae sp.
Laccophilus sp. beetles Beck 1982

Trichoptera

Mystacides alafunbriata caddisfly larvae Abbott Laboratories; Garcia et al. 1980
Several species caddisfly nymphs Abbott Laboratories; Garcia et al. 1980
Limnephilus flavicornis (F.), caddisfly Lebrun and Vlayen 1981
Limnophilus sp. Becker and Margalit 1993
Phryganea sp.

Diptera

Ephydra riparia complex
Dicraneta sp.
Chelifera sp. Abbott Laboratories; Garcia et al. 1980
Musca domestica housefly Vankova 1981; Larget et al. 1981
Procladius freemani
P. sublettei
Tanypus sp. tanypodine midges Mulla et al. 1990
Chanoborus sp. gnat Beck 1982
Drosophila melanogaster fruit fly
Erioischia brassicae cabbage maggot
Toxorhynchites splendens predatory mosquito
Culicoides sp. predatory midge
Chironomus plumosus midge
Lucilia cuprina Akhurst et al. 1997

Plecoptera

Several species stonefly nymphs Garcia et al. 1980; Kreig et al. 1980

Hymenoptera

Apis mellifera honey bee Abbott Laboratories; Garcia et al. 1980
Trichogramma evanescens egg parasite Beck 1982

FLATWORMS

Tubellaria

Dugesia dorotocephala flatworm Abbott Laboratories; Garcia et al. 1980
Dugesia tigrina Becker and Margalit 1993
Bothromesostoma

Platyhelminthes

Dugesia tigrina planarian flatworm Beck 1982

EARTHWORMS

Nadididae

earthworms Abbott Laboratories; Garcia et al. 1980

Lumbricidae

Tubifex sp.
Helobdella stagnalis Oligochaeta
Becker and Margalit 1993; Beck 1982

NEMATODA

Neoaplectana carpocapsae
Heterorhabditis heliothidis entomopathogenic nematodes
Poinar et al. 1990

MOLLUSCS

Gastropoda
Physa sp. freshwater snail
Pelecypoda sp. mussels
Taphius glabratus snail
Abbott Laboratories; Garcia et al. 1980; Larget et al. 1981
Physa acuta
Anisus leucostomus
Bathyomphalus contortus
Hippeutis complanatus
Pisidium sp.
Aplexa hypnorum
Galba palustris
Bithynia tentaculata snail

Planorbis planorbis snail
Radix sp. snail

Vivparus contectus oyster
Ostrea edulis blue mussel
Mytilus edulis moss bladder snail
Becker and Margalit 1993; Beck 1982
Cnidaria
Hydra sp. Becker and Margalit 1993
Rotatoria
Brachionus calyciflorus zooplankton Becker and Margalit 1993

APPENDIX II: Survey Responses

I: Responses from the City of Winnipeg, Canada

QUESTIONS TO AGENCIES USING Bti

1. 1) What type of ecosystem is Bti used in (eg: is it a floodplain wetland, or a more permanent ecosystem)?

   The City of Winnipeg uses Bti in all/any ecosystem types that hold standing water but overall most of our program treats in sites that are flood plains and wetlands.

   2) How much/how often is Bti used in your region?

   2010 – approx 70,000 kilograms and 7300 liters of Bti;
   Program runs daily from mid-April to the end of Sept each year;
   2009 – approx 60,000 kilograms and 5,600 liters of Bti;

2. 3) How is Bti applied (i.e. sprayed as a liquid or spread as a powder)? The City of Winnipeg uses two formulations of Bti; Vectobac 200G (granular on a corn cob substrate) and Vectobac 1200L liquid concentrate mixed with water.

   If a powder, what is the substrate (corn, sand or other)? The City of Winnipeg does not use any Bti powders.

   Do you use a commercial preparation, and what is the name of the product? Yes, Vectobac

3. 4) Do you have a program for monitoring the effectiveness of Bti as a mosquito control agent? Yes, all sites are pre-checked to determine if larvae are present. If present, then treatment of the site occurs by back pack blower, hand, granny gun, helicopter, ATV, ARGO, or gator (converted to carry liquid or granules).

4. 5) Do you have a program for monitoring the effects of Bti on other aquatic taxa, particularly nematoceran Diptera?

We are aware that other Dipterans like the gnats are affected by Bti but with the narrow range of affected non-targets, the City of Winnipeg is increasing its use of Bti and other bacterial based products over the next few years to become fully biological with Bti being the primary control product.

5. 6) Do you have a program for monitoring the effects of Bti on terrestrial biota (eg: terrestrial invertebrate and vertebrate predators, particularly birds)?

   No we do not as the Canadian Pest Management Regulatory Agency and our Provincial Pesticide Use Permit does not make it a requirement for the treatment of standing water sites with mosquito larva with Bti.

6. 7) Do you measure the effects of Bti on any other ecosystem properties (eg: nutrient status, turbidity etc).

   No not a pesticide use permit requirement in Manitoba and/or in Canada

7. 8) If you have a monitoring program, how extensive is it? (number of sites, number of sampling occasions)

   Our program covers an area of approximately 185 square kilometers. In that area approximately 23,000 to 35,000 hectares of surface water area is monitored and treated, when larva are present, on a weekly basis. We have approximately 7,000 listings which equates to tens of thousands of sites that monitored and treated as required each week.

8. 9) If you have a monitoring program, does it include reference (untreated) sites? If so, how were these sites selected

   Yes, we have control sites outside and within our mosquito abatement district to test the effectiveness of Bti on various instars of mosquito larva. The sites are selected based on the same types of characteristics that occur within our MAD, so we have open, grassy, high nutrient/low nutrient load, bush and shaded, permanent, semi permanent and ephemeral.

9. 10) If you have a monitoring program, does it compare treated sites before and after treatment?

Yes, we pre check and post check an average of 50-100 listings each week (random site selection) to determine overall effectiveness of the larviciding treatments with Bti and other larvicides control products.

10. 11) What sampling protocols are used? Which measures exactly are taken?

We collect data on the pretreatment larval levels, larval instar, time/date of treatment, time/date of post check and post dipping larval numbers (24 and 48 hours after treatment) to determine the overall efficacy level.

11. 12) Can you comment on what effects of Bti are seen in your region, both on the mosquitoes and/or on other biota and ecosystem properties?

Bti is a very effective larvicide on 1st – 3rd instars (mostly a 99-100% effective kill rate), in some cases when the larvae are in the 4th instar, the effective application rate needs to be increased to maximum (i.e. 10 Kg/Ha.) to get a reasonable kill of the larvae. It is also temperature dependent as the colder the water site is in the Spring and sometimes late Fall, effective Bti applications on 4th instar can fail at a higher rate than during the summer months. (Based the degree of feeding occurring when water temperatures are not very conducive (cold) for rapid larval development).

12. 13) Can you comment on whether you think the monitoring program is effective in meeting its goals?

It is very successful in meeting our goals at becoming biologically based larviciding program from mostly a chemical program.

13. 14) Are there any publications or reports from the program?

There is an annual report on the entire City of Winnipeg programs overall activities, cost and control successes.

14.15) What approaches do you use in communicating with the public? How conscious are the public of the use of Bti, do they view it positively/negatively? Are they involved in sampling in any way?

The public is extremely supportive of the movement towards the increased use of Bti in so far that the City of Winnipeg has budgeted for a completely biological program with Bti being the primary control product being used (planned for the season in 2012). We will still maintain alternative adult nuisance mosquito control with permethrins and adult control with Malathion 95 ULV

II: Responses from *Le Tour du Valat*, France

QUESTIONS TO AGENCIES USING Bti

1) What type of ecosystem is Bti used in (eg: is it a floodplain wetland, or a more permanent ecosystem)? Temporary salt marshes, permanent and semi-permanent brackish and freshwater marshes with a belt of tall emergent plants (common reed, club-rush, etc)
2) How much/how often is Bti used in your region? From 30 to 50 aerial spraying per year cumulating about 5000 ha of treatment over a 2500 treated area + unknown number of ground sprayings
3) How is Bti applied (i.e. sprayed as a liquid or spread as a powder)? If a powder, what is the substrate (corn, sand or other)? Do you use a commercial preparation, and what is the name of the product? Aqueous solution of Vectobac at 2.5L/ha (but with multiple passages increasing the dosage locally)
4) Do you have a program for monitoring the effectiveness of Bti as a mosquito control agent? yes
5) Do you have a program for monitoring the effects of Bti on other aquatic taxa, particularly nematoceran Diptera? yes
6) Do you have a program for monitoring the effects of Bti on terrestrial biota (eg: terrestrial invertebrate and vertebrate predators, particularly birds)? yes
7) Do you measure the effects of Bti on any other ecosystem properties (eg: nutrient status, turbidity etc). algae density in marshes
8) If you have a monitoring program, how extensive is it? (number of sites, number of sampling occasions): 120 000 euros, 6 partners, topics covered: direct perturbations caused by mosquito-control operation in a natural reserve open to the public on human activities, resting ducks, tree-nesting herons, etc), relative abundance of Odonata along observation transect, density of chironomids and algae in marshes, relative abundance (sweep-

net samples) of reed-dewlling invertebrates, foraging activity and breeding success of bats and birds (house martin). Most of these studies are based on the comparison of 2-6 treated sites with untreated sites with a sampling frequencies adapted to the phenology of the habitats/species studied.

9) If you have a monitoring program, does it include reference (untreated) sites? Yes. If so, how were these sites selected. Based on their ecological similarity with treated sites – actually the limiting factor is the number of treated sites since only 1/10 of the mosquito-producing habitats are actually treated.
10) If you have a monitoring program, does it compare treated sites before and after treatment? Yes, especially for the chironomid & algae monitoring.
11) What sampling protocols are used? Which measures exactly are taken? See articles and reports and response in question 8.
12) Can you comment on what effects of Bti are seen in your region, both on the mosquitoes and/or on other biota and ecosystem properties? Locals are happy with a strongly reduced nuisance, impact on chironomids, spiders, dragonflies and birds are significant.
13) Can you comment on whether you think the monitoring program is effective in meeting its goals? Yes, at least some of them.
14) Are there any publications or reports from the program? Yes, all reports (in French) are available at: http://www.parc-camargue.fr/Francais/download.php?categorie_id=108 + the ms attached.
15) What approaches do you use in communicating with the public? How conscious are the public of the use of Bti, do they view it positively/negatively? The monitoring also involve a sociological study with interviews. People feelings are mixed, they would like to get rid of mosquitoes in inhabitated-areas without impacting nature. Are they involved in sampling in any way? Some people are involved for measuring the degree of nuisance and its variation over the season. Otherwise, most

communication has rather been limited so far because this mosquito-control is a 5-yr experiment and the coordinator is waiting for the final year to communicate the results and decide on what to do next (stop, maintain or expand the currently mosquito-controlled area).

APPENDIX III: GIS map showing location of potential reference wetlands.

Geografiska Informations System (GIS) har använts för att identifiera potentiella referensområden utanför de områden som fram till 2010 har behandlats med Bti. Dalälvens sjösystem uppdelades i 7 delar utifrån höjddata. För att ta fram områden som kan påverkas av översvämning gjordes en urval av områden med en höjd max 1 m över sjöytorna. Ett urval av områden med TWI (terrain wetness index) över 0 gjordes och kombinerades med de beräknade översvämningsområdena, och myrmarker från kartinformation. Ur detta material och med hjälp av tidigare provplatser gjordes ett urval av nya områden där övervakningsstationer skulle kunna läggas. Områdena jämfördes visuellt med Google Maps kartor som i området håller en hög kvalitet, och utifrån dessa kunde ett antal platser som även ligger lättillgängliga väljas ut. Utifrån flygbilderna i Google Maps kunde flera områden avfärdas och några läggas till som intressanta. På detta sätt har 20 olika lokaler/områden identifierats som kan fungera som referensområden, då de är känsliga för översvämningar (Figure 1, nästa sida).

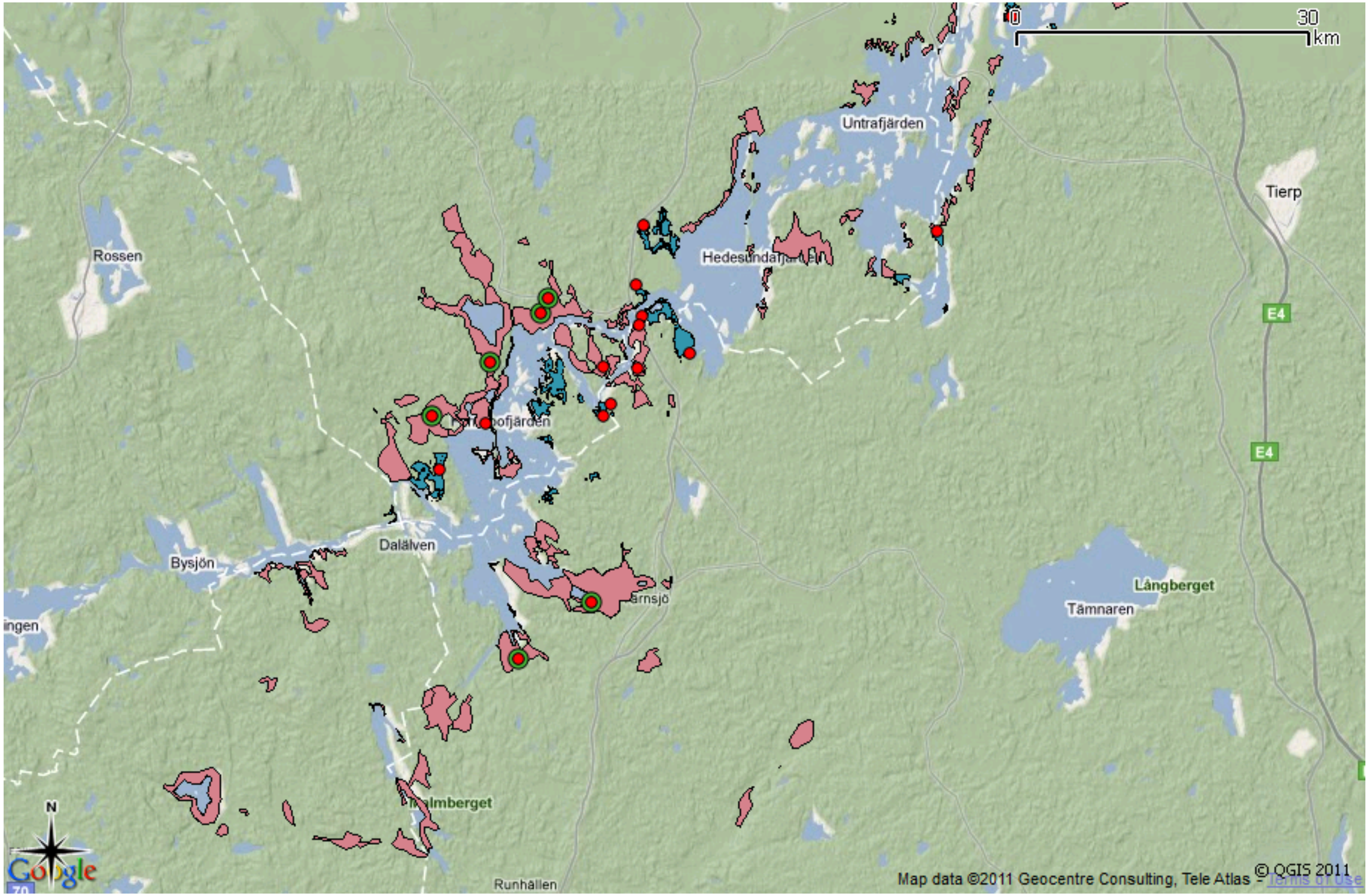

Figure 1: Förslag på övervakningslokaler, baserade på GIS kart anläggning
Legend: Röda områden var behandlade med Bti under 2010; Blåa områden är potentiella referensområden, som inte har behandlats med Bti tidigare, men som har potentiella för översvämningsmygg; Gröna stora prickar är befintliga egenkontroller; Röda mindre prickar är lokaler som besöktes av personal från SLU för rekognosering under höst 2010.

APPENDIX IV: Preliminary suggestions for the biomonitoring of the effects of the mosquito control agent Bti on ecosystems of the Dalälven catchment

Delivered to the Swedish EPA on the 27th November, 2010

(In Swedish)

UPPDRAGET

NV har uppdragit till SLU att utreda pågående övervakning av effekter på miljön av Bti-användningen i Nedre Dalälven. Målet med forskningsutredningen är att ta fram en rigorös och statistiskt fullgod uppföljningsdesign som mäter direkta effekter på målorganismen och icke-målorganismer såsom fjädermygg (chironomider) . . . Även näringstillståndet (övergödning) i vattnet har betydelse för myggens tillväxt, så en bedömning av bekämpningens effekt på näringsstatusen bör övervägas som del i programmet.”

TIDIGARE ÖVERVAKNING 2002-2007: Under perioden 2002-2007 har CO2/ljusfällor och kläckningsfällor användes för provtagning av vuxna mål- och icke-målorganismer. Programmet har haft god tidsmässig täckning (veckovis provtagning under översvämningsperioden), men mycket begränsad geografisk täckning, då endast fyra kläckfällor från vardera av sex översvämningsområden har använts. De sex översvämningsområdena har delats in i tre referensområden (ingen Bti-behandling) och tre behandlade områden (ett av sumpskogskaraktär med dominans av klibbal och två av ängskaraktär med starr och örtvegetation).

BÄTTRE METODER FÖR UPPFÖLJNING AV BEKÄMPNINGENS EFFEKTER

A) Överväganden

- Nedre Dalälvens miljö, med återkommande vattenståndsvariationer och en mångfald av naturtyper, kännetecknas av en extrem rumslig och tidsmässig variation. Till exempel bidrar olika vegetations- och jordartstyper, samt en småskalig topografisk variation, till en stor variation i förekomsten och tätheten (t.ex. antal per m^2) av både målorganismer och icke målorganismer. Denna stora variation försvårar möjligheten att urskilja eventuella effekter av Bti på icke-målorganismer. För att statistiskt kunna säkerställa eventuella effekter av Bti, behöver ett framtida övervakningsprogram därför (i) öka antalet övervakade översvämningsområden och (ii) öka antalet prov som tas inom varje översvämningsområde.

Vidare förslås ett övervakningsprogram tillämpa ett s.k. "*pooling och subsampling*" protokoll i provhanteringen. Detta innebär att samtliga delprov från ett översvämningsområde slås samman (*poolas*) och att sedan ett förutbestämt antal individer, vanligen mellan 300-700, slumpvis plockas ut från det sammanslagna provet. Ett sådant protokoll minskar kostnaden för provanalys, men bibehåller en god täckning av variationen inom varje översvämningsområde. Dylika ansatser är väl etablerade inom biologisk miljöövervakning.

För att ytterligare öka möjligheten att kunna säkerställa en påverkan av Bti på ekosystemen rekommenderas att man i ett framtida övervakningsprogram (i) åtskiljer provtagning av de akvatiska och terrestra faserna av översvämningsområdena, (ii) stratifierar provtagningen för de två viktigaste typerna av översvämningsområden, d.v.s. sådana som är av sumpskogskaraktär med dominans av klibbal och sådana av ängskaraktär med starr och örtvegetation, samt (iii) inkluderar fysikaliska och vattenkemiska mätvariabler.

Lokalisering av flera lämpliga referensområden är viktig och bör prioriteras. Man måste också säkerställa att dessa referensområden förblir obehandlade i ett längre tidsperspektiv, dels för den långsiktiga miljöövervakningen och för studier av långsiktiga effekter av Bti på ekosystemen.

Vidare bör man överväga att ta ytterligare bottenfaunaprov, till exempel med rörhämtare, då denna metod har visat sig vara framgångsrik i andra länder för att upptäcka Bti effekter.

B) Preliminärt förslag och kostnadsuppskattning

Nedan redovisas två förslag till ett framtida miljöövervakningsprogram för effekter av Bti, ett basprogram som endast täcker en typ av översvämningsområden och enbart akvatiska organismer, samt ett utökat program där båda dominerande typer av översvämningsområden, samt kompletterande provtagning av bottenfauna och terrestra fjädermyggor ingår. Kostnadsuppskattningen för båda förslagen nedan är baserad på kostnaderna per prov för den nationella miljöövervakning som SLU bedriver i uppdrag av NV.

1) Basprogram – Provtagning görs bara i översvämmade områden (> 1 cm ytvatten) och bara i behandlade översvämningsområden av ängskaraktär. Minst 5 obehandlade referensområden och 5-10 behandlade områden ingår.

- *Icke-mälorganismer* provtas med 10 kläckfällor per område och provtagningsdatum. Varje fälla exponeras i 48 timmar. Fällorna omplaceras slumpmässigt varje vecka under den mest dynamisk perioden (6-8 veckor när vattennivån förändras fort), annars varannan vecka. Inledningsvis ska fällorna placeras över hela det översvämmade området, för att sedan placeras över kvarvarande vattensamlingar när vattenståndet är lägre.
- *Målorganismer* provtas med 10 ljusfällor per område, installerade samtidigt som kläckfällorna och slumpmässigt fördelade över områdena. Varje fälla exponeras i 48 timmar.
- *Fysikalisk-kemiska variabler* – Vattenprover (5 per område) tas på varje kläckprovningsdatum och analyseras med avseende på närsalter och andra basvariabler. Temperatur loggers (3-5 per våtmark) kan lämpligen appliceras under utvalda kläckningsfälla. Vattendjup intill varje kläckfälla mäts vid varje tömning.

Preliminär kostnadsuppskattning för basprogrammet (12 områden, 12 provdatum) landar på en årlig kostnad på 1,4 miljoner kronor. Av detta går ca 15% till fältarbete, 15% till utrustning, 50% till provbehandling och 20% till vattenkemiska analyser.

2) Utökat program – Provtagning omfattar förutom det som beskrivs i basprogrammet ovan (i) även översvämningsområden som är av sumpskogskaraktär (3-5 behandlade och referensområden, beroende på tillgänglighet), (ii) ytterligare kläckfällor för att provta terrestra fjädermyggor som förekommer i fuktig mark (ytvatten <1cm), samt (iii) bottenfaunaprov tagna med rörhämtare, med sammanslagning och uppdelning av replikat, såsom i bearbetning av prover från kläcknings- och ljusfällor.

Preliminär kostnadsuppskattning för det utökade programmet (20 områden, 12 prov per datum) landar på en årlig kostnad på mellan 2,0 (utan bottenfaunaprovtagning) och 2,5 miljoner.

C) Kommentarer Förslaget måste betraktas som preliminärt. Det finns ett stort behov av en djupare analys av befintliga data, till exempel för att optimera antalet stickprov från varje område som behövs för en god statistisk styrka och för att kvantifiera antalet individer som behöver sorteras från de sammanslagna delproverna. Dessutom saknas grundläggande information om responsen från icke-målorganismer på översvämningar, om den småskaliga fördelningen av icke-målorganismer, samt om hur näring som härstammar från det stora antalet döda stickmyggor och majskornen som följer med Bti-appliceringen påverkar ekosystemen. Ett nytt övervakningsprogram kommer därför att initialt behöva genomgå en optimeringsfas. Andra, mer komplexa, frågor besvaras lämpligen av olika forskningsinsatser knuten till Nedre Dalälvsområdet.

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